

Laboratory of Radiation Biology and Experimental Radiation Oncology

Head: Prof. Dr. E. Dikomey

Oncology Center

Department of Radiation Therapy and Radiation Oncology

Director: Prof. Dr. Cordula Petersen

University Medical Center Hamburg-Eppendorf

**The role of ATR mediated p53 phosphorylation at Serine 15
in Homologous Recombination**

Dissertation

Thesis to earn a Doctoral Degree of Medicine
at the Faculty of Medicine of the University of Hamburg

Submitted by:

Sarah Juliane Lachmayer
born in Moenchengladbach

Hamburg 2010

Accepted by the Faculty of Medicine
of the University of Hamburg on:

July 8th, 2011

Published with the Authority of
the Faculty of Medicine of the University of Hamburg

Examination Board, Chairman:

Prof. Dr. J. Dahm-Daphi

Examination Board, 2nd Consultant:

PD Dr. S. Klutmann

Examination Board, 3rd Consultant:

Prof. Dr. E. Dikomey

Table of Contents

1. Hypothesis	5
2. Introduction	6
2.1. P53.....	6
2.1.1. P53 structure and functional domains.....	6
2.1.2. P53 regulation.....	7
2.1.3. P53 transactivation-independent functions, DNA repair.....	9
2.1.4. The role of phospho-S15-p53.....	9
2.2. ATR.....	10
2.3. Homologous recombination.....	12
2.3.1. Homologous recombination at sites of DSB.....	13
2.3.2. Homologous recombination at sites of stalled replication forks.....	14
2.3.3. Regulation of homologous recombination via p53 and ATR.....	16
3. Materials and Methods	17
3.1. Cell lines.....	17
3.1.1. Plasmids.....	17
3.1.2. Cell culture and maintenance.....	18
3.2. Plasmid amplification and characterization.....	19
3.2.1. Amplification.....	19
3.2.2. Ethanol precipitation.....	20
3.2.3. Characterization of the plasmids.....	20
3.3. Lipofection of the plasmids into the H1299 host cell line.....	21
3.4. Hydroxyurea, thymidine and caffeine.....	21
3.5. Cell fixing and immunostaining for Rad 51.....	22
3.5.1. Immunostaining for γ -H2AX foci.....	23
3.5.2. Foci counting and pictures.....	23
3.6. Complete list of all used materials, reagents and appliances.....	24
4. Results	28
4.1. Establishing isogenic H1299 cell clones with different p53 status.....	28
4.2. Induction of Rad51 foci in response to replication fork stalling.....	30
4.3. The S-15 site of p53 is required for suppression of Rad51 foci formation.....	32
4.4. Time courses of Rad51 foci formation.....	34
4.5. Differential effects of caffeine upon Rad51 foci formation.....	37

5. Discussion	39
5.1. Transactivation-inactive H1299 L22Q, W23S p53 mutant cells retain their ability to suppress HR.....	40
5.2. The S-15 site of p53 is required for HR downregulation.....	40
5.3. HR levels increase after release from TdR induced replication fork stalling...	42
5.4 Low concentrations of caffeine lead to impaired HR suppression.....	45
5.5 High concentrations of caffeine lead to complete inhibition of Rad51 foci formation and HR	46
6. Summary	50
7. Literature	52
8. Acknowledgements	65
9. Curriculum Vitae	66
10. Statue of Originality	67

1. Hypothesis

Clinical heterogeneity in the response of human cancers to DNA damaging agents is one of the leading problems in oncology and cancer treatment. Much of this heterogeneity is caused by genetic alterations that affect the way tumors respond to and repair DNA damage (145). It is therefore crucial to gain a thorough understanding of the cellular damage response at molecular levels, in order to develop individualized therapies that target altered DNA repair pathways in cancer (41).

Homologous recombination (HR) is known to be one of the cells most important repair pathways in response to DNA damage, for instance caused by irradiation or DNA damaging chemical agents. Hyper- and hypoactivity of HR can lead to either elevated rates of crossing over and deleterious or missense mutations of recombination intermediates, or to poor and insufficient repair of DSB. Therefore, strict and highly elaborated regulation mechanisms are necessary to prevent cells from translocations, deletions, duplications, or loss of heterozygosity and subsequent transformation into cancer cells (5). Considering the potential damage caused by excessive HR, the downregulation mechanisms of HR have been proposed to be an important additional system for the cell to prevent carcinogenesis.

Even though proteins promoting HR are well studied, proteins that possibly downregulate HR are poorly characterized at present. In yeast, the ATR (Ataxia teleangiectasia mutated and Rad3 related) homologue Mec1 is anti-recombinogenic in response to replication fork stalling, but whether the same holds true in mammalian cells is still controversially discussed. The tumor suppressor p53 has been described and established as a downregulator for HR, but the exact mechanism is still unknown and subject of investigation.

This study addresses the importance of the interaction of p53 with ATR, testing the hypothesis that phosphorylation of p53 at serine 15 (S-15) by an ATR dependent mechanism is required for the downregulation of HR in response to replicative stress, thereby suppressing HR. An isogenic cell model consisting of 3 variants, one p53null, one p53 transactivation-inactive and one p53 transactivation-inactive and S15A mutated, all three originating from the non-small cell lung cancer cell line NCI-H1299, was used to investigate differences in p53/ATR-dependent HR regulation. Subsequent changes in HR activity were quantified by monitoring the accumulation of Rad51 protein after replication fork stalling.

2. Introduction

The p53 tumor suppressor is one of the most frequently mutated genes in cancer (16). The mechanisms by which p53 affects the cellular DNA damage response have been investigated for years, focusing on p53 function as a transcription factor. However, more recent studies gave evidence that p53 further engages in transactivation-independent functions in DNA metabolism, such as the downregulation of HR (11,17,71,147). HR is an important repair mechanism required for DNA double-strand break (DSB) repair and for the response to replication fork stalling (53). HR levels inside cells are subject to careful regulation by pro-recombinogenic proteins, as well as anti-recombinogenic factors (121,135). Thus, elucidating the role of p53 in HR is of high importance to gain a better understanding of the cytotoxic effects caused by DNA damaging processes.

2.1 P53

The tumor suppressor protein p53 mainly acts as a transcriptional activator or repressor and is involved in the regulation and control of the majority of all molecular pathways contributing to the genomic stability of the cell, such as error free cell cycle progression and division as well as DNA repair and apoptosis (59,64,68,69,120). It is mutated in over 50% of all known types of human cancer (16,29,55) and has therefore been subject of intense study and investigation for the past 20 years.

2.1.1. P53 structure and functional domains

The p53 tumor suppressor gene is located on the short arm of chromosome 17 and encodes for the p53 protein, which consists of 393 amino acids. The protein structure is commonly divided into three functional domains: the N-terminal domain, the C-terminal domain and the intermediate Core-domain. The N-terminal domain mainly consists of the transactivation domain and the proline-rich SH3 (Src homology 3-like) site (Fig.1). The transactivation domain is subject to phosphorylation by various protein-kinases and is required for the regulation of transcriptional activity, interaction with different transcription factors and the MDM2 (mouse-double-minute) ubiquitin ligase.

2. Introduction

The SH3 site interacts with proteins protecting p53 from degradation (SIN3).

The Core-domain of p53 is highly conserved and analogous in all species. It contains the DNA double-strand binding site which is essential for adequate p53 binding to gene promoters and other regulative DNA elements. Its crucial role for p53's transactivation function as well as its size predestine the core domain as a mutation 'hotspot' region in the p53 gene with more than 85% of all known mutations located between AA 105 and 292. (51,92). The C-terminal domain consists of the nuclear localization and export signals, the tetramerization domain and the C-terminal regulatory domain. It is further capable of recognizing multiple DNA lesions, e.g. single stranded ends, mismatches, insertion/deletion or recombination intermediates (7,34,66).

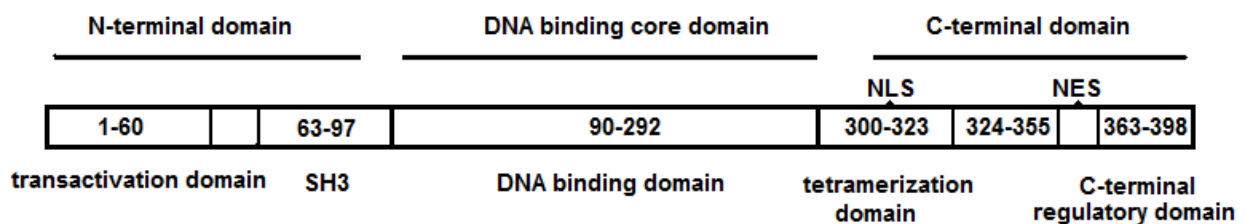


Figure 1: Model of the functional domains of p53.

2.1.2. P53 Regulation

P53 is constitutively present at low levels in the cytoplasm of mammalian cells and downregulated via interaction with the E3 ubiquitin ligase MDM2, which in turn is transcriptionally activated by p53 in an autoregulatory loop (98,149). The continuous ubiquitylation via MDM2 leads to subsequent degradation of p53 in proteasomes (24,83,149). In response to DNA damage (UV, ionizing radiation, oxidative stress, chemical agents, hypoxia,) and replicative stress, p53 downregulation is suppressed. The protein is activated and stabilized (44,85) leading to accumulation, translocation to the nucleus and protein tetramerization (43,82,112). Tetramerization is the crucial step that enables p53 to act as a transcription factor via binding to regulative DNA elements of target genes. Transcriptionally active p53 has so far been identified as a specific activator or repressor for over 150 genes, mainly targeting cell cycle control,

2. Introduction

growth, apoptosis and DNA repair to maintain genomic stability and prevent deleterious cell cycle progression. After initial activation, p53 is subject to an array of posttranslational covalent modifications, primarily site-specific acetylation, phosphorylation and dephosphorylation (Fig. 2), which tightly regulate its transactivation and transactivation-independent functions (16,56,119). A growing number of these phosphorylation sites are identified and their purpose has partially been cleared (16,20,31,87), while other phosphorylation sites as well as the corresponding kinases are still under investigation .

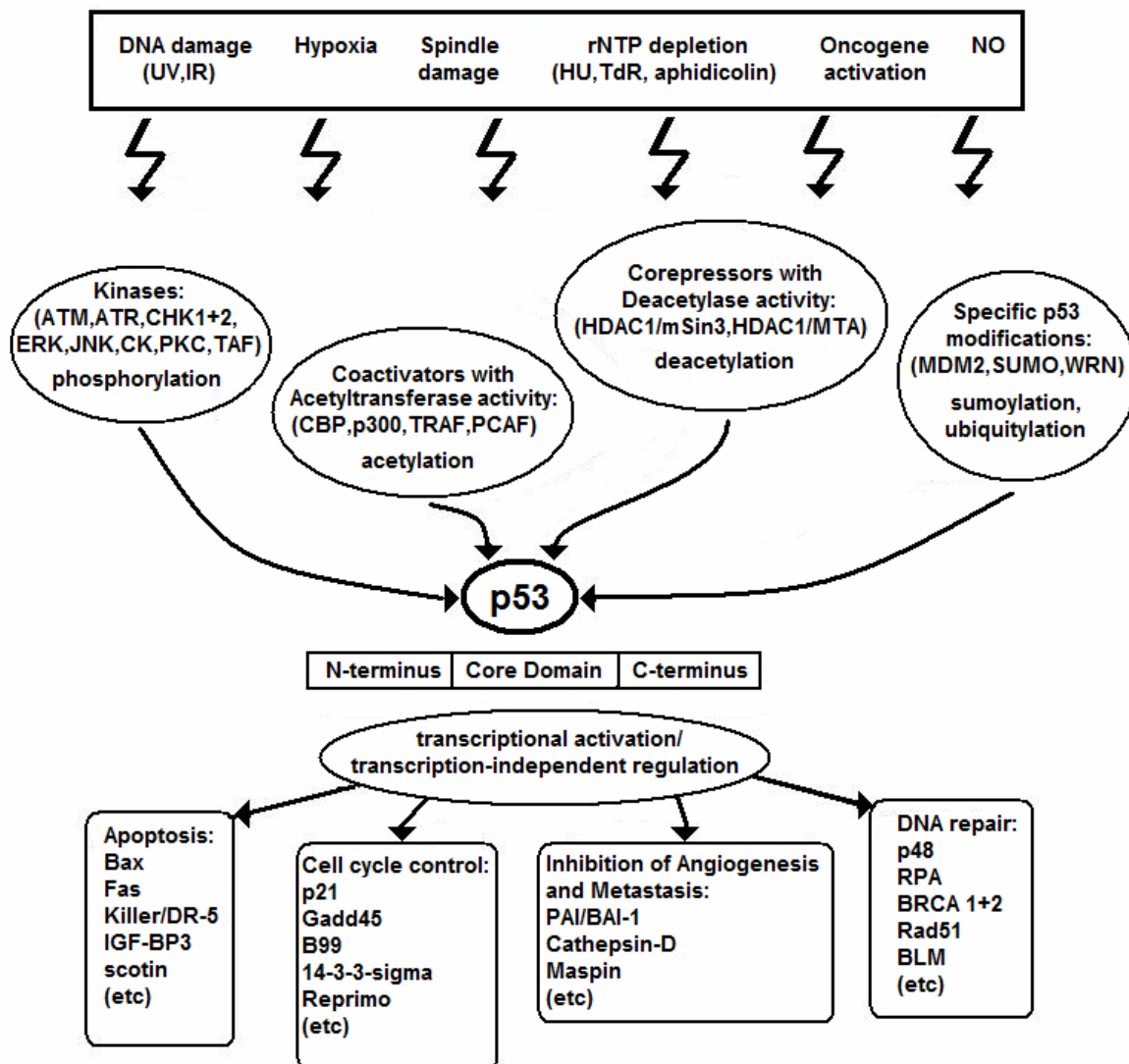


Figure 2: Model of p53 upstream and downstream regulation.

2. Introduction

2.1.3 P53 transactivation-independent functions, DNA repair

Over recent years, the established theories about p53 pathways and functions have extended beyond its traditional role as a mere transcriptional activator.

The N-terminal p53 mutant L22Q, W23S has become a valuable tool to study transactivation-independent p53 functions (137), as it abrogates p53 binding to specific gene promoters, e.g. the respective TATA boxes. Transactivation-independent functions of p53 include apoptotic and cell cycle regulatory functions (104), but have been best described for its involvement in DNA repair pathways (16,100). In general, the presence of excessive and severe DNA damage leads to p53 mediated induction of cell cycle arrest to provide adequate time for successful and error-free DNA repair. If the damage exceeds the capability of the repair mechanisms, p53, in cooperation with other regulatory proteins (e.g. BLM), induces cell death via apoptosis (141). If adequate DNA repair is possible and required, p53 is able to recognize and bind to damaged (single-stranded) DNA segments via its C-terminal domain (61,73) and to mediate DNA repair via direct protein interactions with kinases and enzymes involved in the respective pathways (11,31,48,88). Direct or transactivation-independent p53 functions in DNA repair are exerted in nucleotide excision repair (p48, XPC and TFIIH) (58,143), base excision repair (APE/REF1, DNA polymerase β , OGG1) (2,160), mismatch repair (MSH2, Mre11) (116), non-homologous end joining and homologous recombination (see below).

2.1.4. The role of phospho-S15-p53

In response to DNA damage accomplished via irradiation, cisplatin or replication fork stalling (via hydroxyurea, thymidine or aphidicolin), p53 is phosphorylated at N-terminal serine residue S-15 by the PIKK kinases ATM and ATR (4,50,111,134). This phosphorylation was formerly thought to be only required for p53 stabilization and accumulation after DNA damage, partly due to impaired binding and interaction with MDM2 kinase (122), indicating a rather passive effect of S-15 phosphorylation on p53 regulation. However, impairment of MDM2 interactions has not only been reported for S-15, but also for serine residues 37, 20 as well as threonine 18 (27), suggesting additional purposes for S-15 apart from plain p53 regulation. In further studies, the phosphorylation has been found to result in direct activation of p53 (111,126),

2. Introduction

facilitating initiation and regulatory involvement in apoptotic processes (124) as well as enhanced binding to p300, which increases p53 transcriptional activity via acetylation (32). Interestingly, functionally impaired mutant S-15 p53 has been found to retain its transactivation activities (46), suggesting that the purpose of S-15 phosphorylation is not exclusively limited to its transactivation activities. S-15 phosphorylation has further been shown to be required for subsequent acetylation at specific C-terminal residues that are essential for recognition of specific sites of DNA damage (111).

Focusing on p53's involvement in DNA repair pathways, phospho-S-15-p53 has been demonstrated to associate with DNA recombination repair complexes (121,161) and to co-localize, associate and directly interact with key enzymes of homologous recombination repair such as MRE11, Rad51 and Rad52 (71,103). These observations shed new light on p53's involvement in DNA repair, not only as an indirect activator of DNA repair pathways via transactivation, but also as an active and direct mediator for the respective pathways.

2.2. ATR

ATR (ATM and rad3-related) is a large, >300kDa protein, and a member of the PIKK (phosphoinositide 3-kinase-like kinase) family, a small nuclear group of Ser/Thr kinases which is structurally related to the superfamily of PI-3 kinases. Members of this family (ATM, ATR, DNA-PK, m-TOR, SMG-1) and their downstream proteins are activated in response to replicative stress and are involved in the regulation of cell cycle checkpoint control, DNA repair and apoptosis (1,25,35,123).

In contrast to ATM and DNA-PK, which are activated in response to DNA double strand breaks, ATR is preferably activated in response to UV induced damage or replicative stress, such as dNTP (deoxynucleosidetriphosphate) depletion via hydroxurea (HU), Thymidine (TdR) or inhibition of polymerase α via aphidicolin treatment (1,6,35,136). DNA lesions that persist during replication cause functional uncoupling between blocked DNA polymerases and helicases which continue to unwind the DNA strand, resulting in long stretches of ssDNA. Those ssDNA stretches induce the recruitment of the ATR/ATRIP complex to sites of DNA lesions (162).

2. Introduction

ATRIP (ATR interacting protein) is a small protein that exclusively heterodimerizes with ATR and facilitates stabilization, proper DNA binding and interactions with other proteins (26,162).

In addition to ATR/ATRIP, a number of other proteins (claspin, TopBP1, Rad17-Rfc2-5, 9-1-1-complex) is recruited to damaged DNA sites (37,60,63,76,163,). These proteins are necessary to facilitate phosphorylation and activation of Chk1, the major downstream protein of ATR (72,157). Chk1 is a key enzyme for initiation and regulation of the intra-S, S/M and G₂M checkpoints. Activation of Chk1 during S-phase leads to stabilization of replication forks, downregulation of late origin firing, blocking of cell cycle progression and facilitation of replication fork restart (86,114,158,80) (Fig.3).

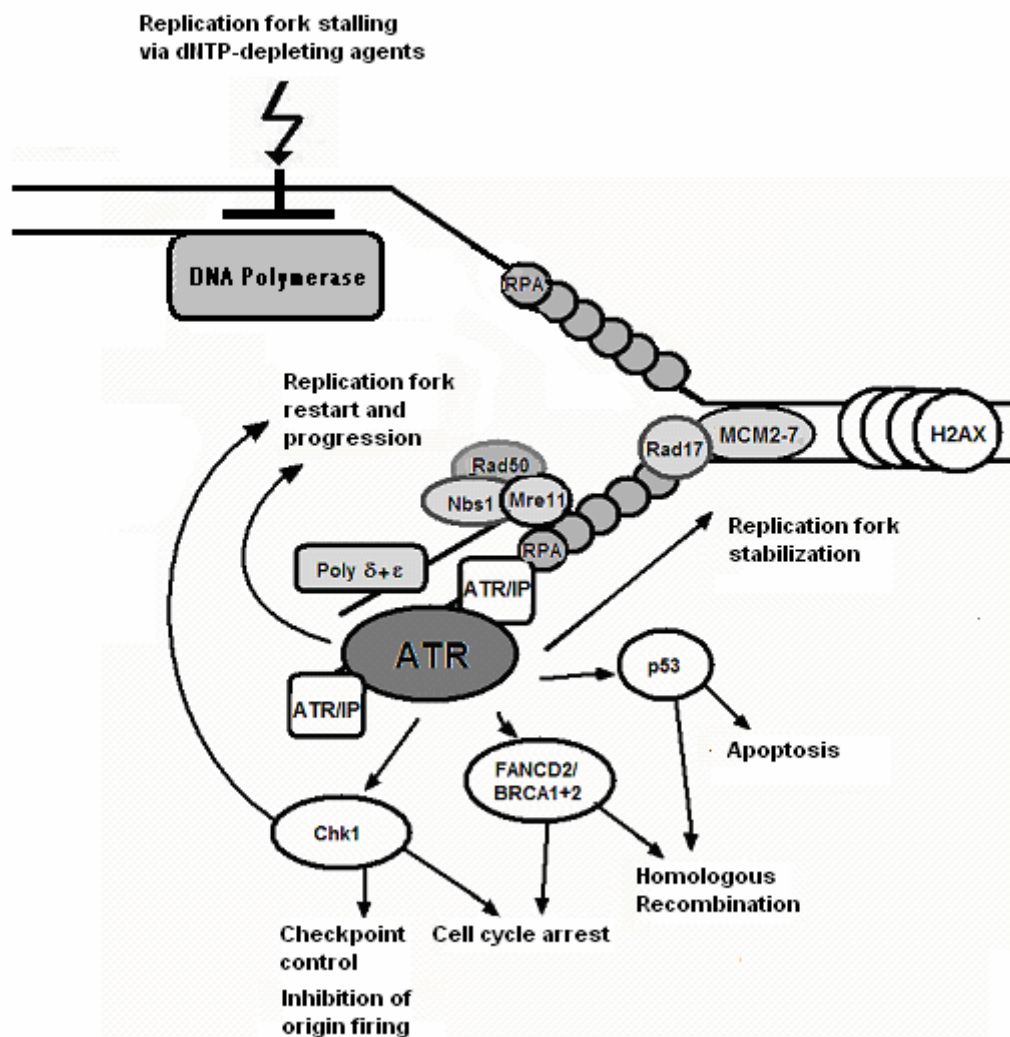


Figure 3: Model of ATR mediations at sites of stalled replication forks.

2. Introduction

Besides its crucial function in replication fork stabilization and cell cycle checkpoint control, ATR directly phosphorylates a large number of proteins involved in several DNA repair pathways. Identified target proteins include BRCA1, BLM, WRN (participating in HR and NHEJ), FANCD2 (crosslink repair), and XPA (NER) (3,28,70, 95,133,149). In response to DNA damage caused by UV irradiation or stalling of replication forks, ATR acts as an upstream regulator for the tumor suppressor protein p53 (134), phosphorylating p53 at N-terminal sites S-15 and S37 which in turn are involved in protein stabilization, DNA binding, apoptosis (79,129,134,162) and interaction with several replication proteins (71,103) (see below).

2.3. Homologous Recombination

Homologous recombination (HR) is a highly conservative and tightly regulated DNA repair process in all known species and is activated in response to various types of DNA damage, e.g. DSBs, interstrand crosslinks and replication fork stalling in S-phase. It is conducted via DNA single strand invasion into a homologous sequence of the genome, preferably located on the sister chromatid. The homologous sequence is then used as a template to copy the correct genetic information. Similarities in procedure, participating enzymes and their molecular structure in bacterial and yeast organisms compared to human cells helped to understand the underlying mechanisms of HR in vivo. Repair enzymes such as BRCA1, BRCA2, FANCD2, BLM, ATR, p53 and Rad51 drive HR directly or participate at various up- and downstream levels. All steps of HR and the participating enzymes are tightly controlled via up- and downregulation to generate equalized levels of HR in cells. Failure to maintain this carefully regulated HR equilibrium leads to either insufficient or excessive DNA repair. Both conditions are disastrous for cell viability, resulting in various types of mutations like deletions, translocations, duplications, loss of heterozygosity or aneuploidy (67,159). Protection from inappropriate HR therefore contributes to genomic stability and can prevent mutations and malignant transformation.

2. Introduction

2.3.1. Homologous recombination at sites of DSBs

Initiation of HR at sites of DSB is carried out via a range of protein interactions. In the first step, sites of DNA DSB's are detected by the MRN complex (consisting of the DNA damage sense kinases Mre11, Rad50 and Nbs1), followed by resection in 5'-3' direction via the complex' exonuclease activity in order to generate single stranded DNA ends (90). Free ssDNA is immediately coated by RPA (replication protein A) which on one hand supports HR by stabilizing ssDNA and preventing secondary structures, and on the other hand strongly inhibits association of Rad51 (36,132). Rad51 is the key enzyme and core mediator of HR, its recruitment and binding to sites of damaged DNA are crucial for successful HR conduction. Association of Rad51 to ssDNA and interaction with RPA to release inhibitory effects is mediated via several Rad51 cofactor proteins collectively called mediators. The group of mediators in human cells includes BRCA1, BRCA2, Rad52 and the Rad51 paralogs Rad51B, Rad51C, Rad51D, Xrcc2 and Xrcc3, all of which have been reported to be essential for Rad51 and RPA interaction (125,131,152). After dissociation of RPA, Rad51 forms long nucleoprotein filaments at the 3' end which, supported by Rad54, facilitate search for homologous sequences, subsequent strand invasion into the unwound D-loop and strand transfer, the central step of HR (also called synapsis) (9,94). The newly bound 3' end serves as primer for DNA synthesis beyond the damaged site. In the currently favored model of HR, elongation of the migrated strand and further unwinding of the template lead to Rad52 mediated invasion of the second end of the DSB (130). The resulting structure model is called double Holliday junction. After successful repair, Holliday junctions can generally be resolved via two pathways, either by dissolution into obligatory non-crossing over products by BLM and Topoisomerase III, or by cleavage via specific resolvases, which may lead to crossing over or non-crossing over products, depending on the direction of cutting.

However, both pathways of Holliday junction cleavage result in exchange of genetic information, known as gene conversion.

2.3.2. Homologous recombination at sites of stalled replication forks

HR at sites of stalled replication forks is restricted to the S-phase of the cell cycle, a time point when the cells DNA is usually highly vulnerable to damage. Minor DNA lesions which may usually be tolerated by the cell during other cell cycle phases now become replication obstacles to the proceeding replication forks. The repair and restart of the stalled replication forks requires an extremely precise and tightly regulated mechanism of repair. Failure of adequate restart can result in cell cycle arrest and in various types of mutation or loss of genomic material (49,57). The stalling of replication forks typically occurs at sites of DNA damage (e.g.SSBs) and affects either the leading or the lagging strand. The fork is unable to proceed replication at the lesion due to interruption of the reading process of the polymerases and is halted. At the same time helicases may proceed beyond the damage leaving long stretches of ssDNA behind. Digestion of these stretches via nucleases can lead to one-ended DSB with long, overlapping ssDNA ends and no partner strand to join to. This strongly increases the risk of aberrations and, in contrast to other HR-inducing types of DNA damage (UV-irradiation) is also exclusively dependent on HR to restore fork proceeding and to prevent loss of information. HR at sites of stalled replication forks is initiated by ATR/ATRIP, which specifically detects stalled replication forks and stretches of ssDNA (see above) and initiates cell cycle arrest. SsDNA ends are coated and stabilized by RPA. The following steps include fork stabilization, association of Rad51, single-strand invasion into the non-affected double helix and repair synthesis, as described above for HR of frank DSB (Fig. 4). Artificial stalling of replication forks, as conducted in this study, can be achieved via replication fork stalling agents such as thymidine, which is incorporated and transformed into dTTP by the cells. High amounts of dTTP induce a partial inhibition of the ribonucleotide reductase and a dysbalance of the dNTP pool. Reduced availability of dNTPs results in a dysfunction of the DNA polymerase complex and to subsequent replication fork stalling (12,45). The release and restart of the artificially stalled forks is conducted via HR as described above, though this model does not include an initial strand lesion. DSB and SSB have been described to occur during the process of fork restart and are discussed to be a result of Mus81 endonuclease activity and replication fork collapse (see discussion).

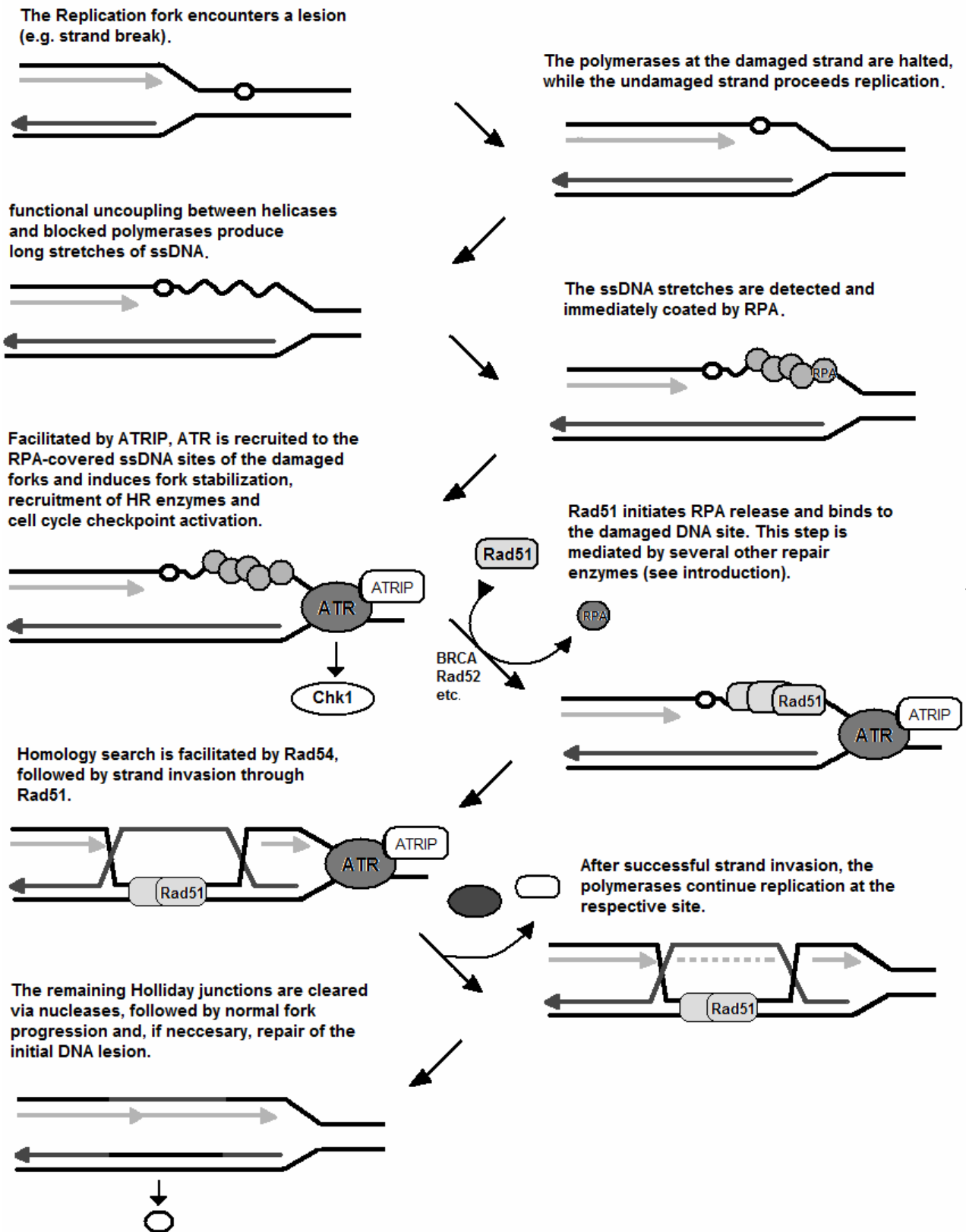


Figure 4: Model of homologous recombination at sites of stalled replication forks.

2.3.3. Regulation of HR via p53 and ATR

HR downregulation has been proposed to serve as a damage prevention mechanism, since HR intermediate structures (ssDNA, Holliday junctions) are highly fragile and vulnerable to breaks and other damage. P53 participates in control and surveillance of HR and has repeatedly been reported to be required for HR downregulation (13,74,110). This has been confirmed in different experimental layouts, concerning both wild-type as well as transactivation-inactive mutant p53 (17,110) (however a structurally intact core domain as well as a functional oligomerization domain of p53 seems to be required for specific HR downregulation (17,33,147)).

P53 interacts with Rad51, the key enzyme of HR and inhibits formation of Rad51 foci during replication (19,65,112,128,153). Other associations and interactions with HR enzymes have been reported for different steps of HR: enzymes involved initially and early in the recombination process such as RPA (106) and Rad54 (5), control and surveillance proteins such as BRCA1 and 2 (77,156), and the replication associated helicases BLM and WRN (150). P53 is further able to recognize and directly bind to stalled replication forks and recombination intermediate structures such as ssDNA or Holliday junctions (73,129).

Taken together, p53 is involved in HR surveillance on multiple levels from recognition of stalled replication forks to initiation, progression and termination of HR.

ATR is activated in response to replication fork stalling (see ATR pathway) and is involved in fork stabilization, cell cycle arrest and facilitation of fork restart. It has therefore been proposed to be an important control enzyme to secure adequate and correct conduction of HR. Once activated in response to replication fork stalling, ATR is able to phosphorylate p53 at N-terminal Serine15. Phospho-S-15-p53 has been reported to transiently or stably associate with key HR enzymes (103) and has recently been shown to directly affect levels of HR in cells (102). Studying the influence of the ATR-dependent S-15-phosphorylation on HR regulation is therefore a promising approach to gain a better understanding of the elaborate mechanisms of HR regulation.

3. Materials and Methods

3.1. Cell lines:

The experiments were conducted using the non-small cell lung carcinoma cell line NCI-H1299 (American Type Culture collection, # CRL-5803). H1299 cells harbor a partial homozygous deletion of both alleles of the p53 gene and are functionally p53 null.

The established subclone H1299 FRT Zeo #1 carries a stably integrated genomic FRT target sequence for the FLP recombinase, enabling targeted integration of a gene of interest into the FRT site.

H1299 FLP QS 22,23 #7 is a FLP-In product of H1299 Zeo, transfected with a transactivation-inactive mutant form of p53 (L22Q, W23S, see below).

H1299 FLP QS 22,23 S15A clone #4, also created via transfection, contains another FLP-in p53 product, which translates for both, the FLP QS mutation and a Serine 15 to Alanine mutation, inactivating the main phosphorylation target site for ATR and ATM.

3.1.1. Plasmids:

The N- Terminal mutant S15A and S20A plasmids for the different cell clones were derived from pRc/CMV5.5 (provided by Dr. A. Dutta). During the course of the study, the S20A plasmid was excluded from the experimental process and used to provide stock solution only.

The pRc/CMV5.5 plasmid itself already harbours the QS 22,23 mutation, consisting of amino acid exchanges at Leucine 22 to Glutamine and Tryptophane 23 to Serine. The additional Serine 15 to Alanine and Serine 20 to Alanine mutations for S15A and S20A plasmids (Fig.5) were introduced via site-directed mutagenesis (Quik Change® II Site-Directed Mutagenesis Kit, Stratagene) according to manufacturer's instructions. Plasmids were then purified (Promega Wizard plus Minipreps DNA Purification System A7510) and stored at -20°C. These plasmids were generated during preceding experiments in this laboratory and were ready to use.

3. Materials and Methods

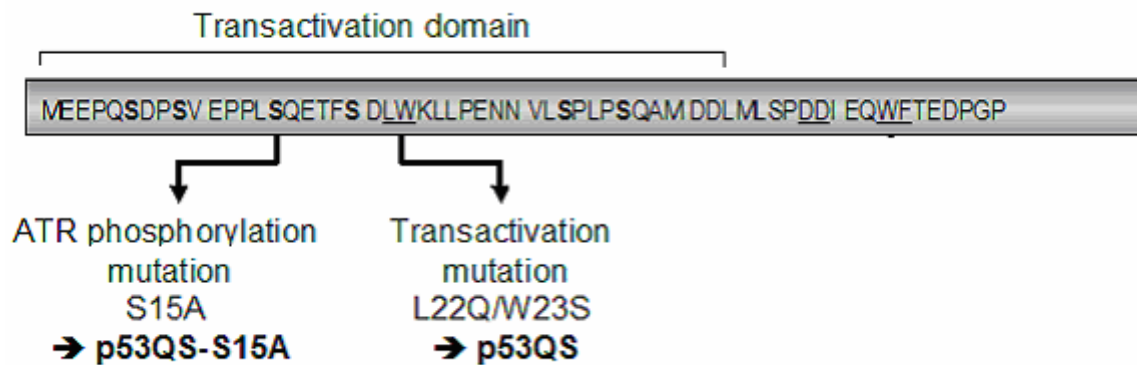


Figure 5: The N-terminal sequence of p53 contains the main transactivation domain. Mutations of L/W 22,23 to Q/S 22,23 generate a transactivation inactive mutant p53. The Serine 15 to Alanin mutation inactivates the main phosphorylation site for the ATR kinase.

3.1.2. Cell culture and maintenance

Cells were maintained at 37°C, 5% CO₂, humidified atmosphere in complete RPMI 1640 medium containing 10%BGS, 10mM HEPES, 2mM/L-Glutamine and 1% Penicillin/ Streptomycin. Regarding the H1299 FRT Zeo cells, 50 µg/ml Zeocin was added to the medium to ensure stable maintenance of the transfected plasmid, for the H1299 FLP QS and H1299 FLP QS S15A lines, 200ug/ml Hygromycin was added. All cell lines were seeded at densities of at least 10⁶ cells/T75 tissue culture flasks and subcultured every two to three days. Cells were used between the passages 5 and 25 to minimize the risk of aging and spontaneous mutation during the course of experiments.

In order to permanently store the cells in liquid nitrogen, confluent cultures were trypsinized and diluted in antibiotic-free RPMI 1640 medium, then counted and spun down at 1000 rpm for 4 minutes. The pellets were resuspended in cooled 10% DMSO/medium at a concentration of 10⁶cells /ml and transferred to 1.5 ml cryo-vials. The vials were placed on ice for 10 minutes, in a -20°C freezer for about 2 hours, transferred to -70°C overnight and then to the liquid nitrogen tanks the next days. If needed, vials were quickly thawed in a 30°C water bath. Cells were spun down in 8ml regular RPMI at 1000 rpm, supernatant including the toxic DMSO was discharged and the cells were resuspended in 4 ml of medium and grown in T25 flask

3. Materials and Methods

for three days until selection medium was added. All cell culture was conducted under sterile conditions in a vertical flow hood.

3.2. Amplification of plasmid and characterization of the mutations L22Q,W23S, S15A and S20A

The plasmids pRc/CMV5.5 L22Q,W23S S15A and pRc/CMV5.5 L22Q,W23S S20A were transfected into E.coli, amplified and harvested using DNA Mini and Maxi extraction kits (Sigma). The DNA was then concentrated via Ethanol precipitation and characterized using certain restriction enzymes for digestion and a gel-run for UV-pictures of the digested plasmid fragments.

3.2.1. Amplification

Both of the Plasmids, concentrated at 1 $\mu\text{g}/\mu\text{l}$ were taken from the provided stock at -20°C , brought to room temperature and slightly vortexed. 2 μl of DNA each were taken out of aliquots and transferred to pre-chilled 1.5 ml Eppendorf tubes to avoid temperature peaks during electro transformation. 20 μl of electro-competent E coli (Coli SURE Electroporation-competent cells, Stratagene) were carefully mixed with 2 μl DNA and electroporated at 1.8 kV and time constant of 4.3ms for S15A and 4.7ms for S20A plasmid. Bacteria were then immediately resolved with 1 ml SOC Medium, transferred into 15 ml centrifuge tubes and placed in a rotating incubator at 225 rpm and 37°C for 1 hour. Cells were then spread in different amounts (5-50 μl) and incubated in shaking incubator at 225 rpm at 37°C for 1 hour on pre-warmed LB-plates with 100 $\mu\text{g}/\text{ml}$ Ampicillin and incubated overnight. Individual colonies were then picked and expanded in fluid LB/Amp medium first for 12h in 250ml, then for 12h in 500ml at 37°C while shaking overnight.

The DNA of both plasmids was harvested from 250ml LB E.coli liquid cultures, using a Gen-elute Maxi prep Kit (Sigma), according to the manufacturer's instructions. Briefly, about 150 ml of each overnight culture was transferred to provided columns, pelleted by spinning down, then resuspended in buffer and lysed for up to 3 minutes. After separating the fluid contents from the aggregated cell remains via neutralization and centrifugation, the DNA-containing supernatant was carefully transferred to another column, cleared twice with endotoxin-removal solution on ice and transferred

3. Materials and Methods

to prepared silica gel DNA-binding columns. High molecular weight bacterial DNA was then washed and centrifuged with washing solution for several times. Plasmid DNA was then eluted in 500µl 60°C, endotoxin-free H₂O.

3.2.2. Ethanol precipitation

To generate a higher concentration of the purified DNA, 3M sodium acetate was added (10% v/v) and vortexed carefully. 2.5 volumes of ice-cold 100% ethanol were added, mixed and stored for 15 min at -70°C. The DNA precipitates were centrifuged at 20.000 x g for 20 min. Supernatant was discarded and the pellets were resolved and washed with 500ul of 70% ethanol. After repeating centrifugation, the pellets were dried at air and dissolved in 500ul ddH₂O, then stored at -20°C.

3.2.3. Characterization of the Plasmids

Both Plasmids were digested with the restriction enzymes Acc1 and Sca1, leading to 3 (1701, 2229 and 2908 bp) and 2 fragments (3754 and 2031bp), respectively. 20µl of plasmid were mixed in a PCR tube with 24µl ddH₂O, 5ul 10x buffer G for ACC1, buffer K for Sca1, and 1µl Acc1 and Sca1 each, and then digested at RT for 2 hours. After digestion, plasmids were mixed with DNA dye (Bromocresol green 0.25%, Xylene cyanol FF 0.25%), mixed with loading buffer(DNA ladder, Invitrogen) and run on a 0.7% agarose gel, supplemented with 0.2µg/ml ethydiumbromide in 1x TBS buffer at 120-140 mV for about 30min. The verification process generated the expected bands at the right cut sizes for S15A and S20A.

3. Materials and Methods

3.3. Lipofection of the Plasmids into the H1299FRT host cell line

2 days prior to transfection the H1299FRT host cells were seeded into 3 T25 flasks. After checking viability and confluency (80%) of cells, the flasks were rinsed with antibiotic-free RPMI 1640 medium and filled with 3.5 ml afterwards. The plasmid DNA of the S15A, S20A or the empty, hygromycin-resistance translating control vector (pcDNA5.5) were each combined at a ratio of 9:1 with pOG44 plasmid and quickly diluted with 300µl of serum-free medium. In 3 different separate tubes, 50µl of Metafectene (Biontex) were mixed with 300µl of serum-free medium, quickly added to the plasmid/pOG44 mixture and incubated at RT for 15 minutes. Thereafter, DNA/lipid complexes were added to the T25 flasks and incubated at 37°C. After 4 hours, medium was replaced by 4ml of fresh medium and cells were incubated overnight. Cells were then trypsinized, counted and plated to 6 cm dishes at concentrations of 4000-6000 cells each plate, after 24 hours changed to medium containing 100µg/ml Hygromycin, and after 4 days to concentrations of 200µg/ml Hygromycin. Medium was changed every 4-5 days.

After 12-14 days, 12 colonies of the S15A and S20A plasmid-transfected colonies each were trypsinized using small plastic cylinders dipped in sterilized Vaseline, then transferred to 12 well plates and expanded in regular RPMI 1640 or Hygromycin selective medium and transferred subsequently to larger well plates and T25 flasks. At the time point the individual cell line clones had expanded to an appropriate number of cells, they were frozen down and stored in liquid nitrogen for further experiments.

3.4. Hydroxyurea, thymidine and caffeine

Prior to treatment, cells were grown to confluence and plated into 8-well chamber slides in non-selective medium at densities of 7×10^4 to 8×10^4 cells/ml, 0.5ml each well, 4 wells each slide, and stored overnight to attach. Concentrations and time for the basic treatments (HU, TdR or CF, 5mM for 24 hrs) were based on preliminary data provided by Bianca Sirbu. For the hydroxyurea treatments, 100ml of a 100mM/ml stock solution in water was prepared, sterile filtered and kept at 4°C. Thymidine and caffeine solutions at 5mM were freshly prepared from 99% powder stock, dissolved in regular RPMI 1640 medium and sterile filtered for every new

3. Materials and Methods

course of experiments.

3.5. Cell fixing and immunostaining for Rad51

After drug treatment, cells were washed 1x with PBS (pH 7.4) and permeabilized for 3 minutes with 0.5% Triton X/20mM HEPES, pH 7.9 /50mM NaCl /3mM KCl /300mM Sucrose. After permeabilization, cells were immediately fixed for 30 min with 3%PFA in PBS/2%Sucrose/1xPBS. Unspecific antibody binding was blocked with 0.1% Triton X/10% BGS for 45 minutes in humidified chamber or stored overnight at 4°C. After washing 3x5 min with PBS, cells were incubated for 3h in a humidified chamber at 37°C with 100uL/well 1:200 primary rabbit anti-Rad51 Ab-1 in 2% BSA/BGS, 1% Triton X-100. Cells were then washed 3x 5 min with 100µL/well 0.1% Triton X/ PBS. Secondary Antibody Alexa Chicken 488 anti-rabbit was also diluted in 2% BSA/BGS, 1% Triton X-100 at a concentration of 1:1000 and incubated for 1hr at RT in a light-proof humidified chamber. After immunostaining, cells were washed 3x5 min with 100uL/well 0.1% Triton X/ PBS, then treated with 10µg/mL DAPI for 2 min, then washed again 2x2 minutes with 0.1% Triton X/PBS. Slides were then separated from the attached wells and dried, applicated with Antifade, covered with cover slips and sealed with nail polish. Slides were counted 16-36 hours after staining.

3. Materials and Methods

3.5.1. Immunostaining for γ -H2AX foci

Double strand breaks were generated via irradiation with 2Gy or after thymidine treatment; the subsequent staining process almost resembled the Rad51 foci staining process, except for antibodies and incubation times. Primary antibody was γ -H2AX mouse monoclonal Ab in 2% BSA/BGS, 1% Triton X-100 at a dilution of 1:100, and was incubated 3-4 hours at 37°C in a humidified chamber. Secondary goat anti-mouse Ab, diluted 1:1000 in 2% BSA/BGS, 1% Triton X-100 was incubated for 2 hours in a light proof humidified chamber at RT. Slides were counted 16-36 hours after staining.

3.5.2. Foci counting

Slides were analyzed via immunofluorescence microscopy at a magnification of 100x, using oil immersion. After checking for apoptotic and damaged nuclei, which tend to exhibit misleading high numbers of foci and therefore had to be excluded from the counting process, the quality and quantity of the remaining foci were determined for 100 nuclei per well. Pictures were taken using exposure times between 4-7ms for DAPI and 0.5-1s for green fluorescence.

3. Materials and Methods

3.6. Complete List of all used Materials, Reagents and appliances

<u>Name</u>	<u>Manufacturer/specifications</u>
<u>Cell culture materials</u>	
RPMI 1640 medium	Sigma /4°C
L-Glutamine	Sigma /aliquoted at -20°C
Penicillin/Streptomycin	Cellgro, #30-002CL /-20°C
Hepes	Siena /4°C
BGS (bovine growth serum)	Hyclone, #30541.03 /4°C
PBS (Phosphate buffered saline)	137 mM NaCl 2,7 mM KCl 2 mM KH ₂ PO ₄ 10 mM Na ₂ HPO ₄ x 2H ₂ O / 4°C
Trypsin	Sigma, Autoclaved Sigma, Trypsin EDTA solution #T4174 /4°C
Hygromycin B	Invitrogen, #10687-010, /4°C
Zeocin	Invitrogen, #46-0509, /-20°C
DMSO	Sigma, #D4540-100ML /RT
FLP-IN System	Invitrogen, Cat # K6010-01
Tissue Culture Flasks,	BD Falcon, 0.2mm vented blue cap 0,2mm vented blue cap
Pipets	Fisherbrand
Tubes	BD Falcon Blue Max Polypropylene Conical Tube
Dishes and 6,12 Well plates	BD
Incubator	Forma Scientific Water jacketed Incubator

3. Materials and Methods

<u>Name</u>	<u>Manufacturer/specifications</u>
Nitrotank	Forma Scientific Cryomed CM290
Centrifuge	Thermo Electron Corporation IEC Central CL2 Centrifuge
Cryotubes	NUNC CryoTube Vials #377267
-20°C, -70°C freezer	NINTA, VWR Scientific
<u>Plasmid Amplification and Characterization</u>	
Bacteria	Stratagene, Epicurion Coli SURE Electroporation-competent cells #200227, / -70°C
Plasmids	in stock, pRc/CMV5.5 QS 22,23 /-20°C pRc/CMV5.5 QS 22,23 S15A/- 20°C
SOC Medium	Invitrogen, #15544-034 / RT
LB Medium and Plates:	
Bacto-tryptone	BD, #225930
Bacto-yeast extract	BD, #212750
NaCl	Sigma, #S7653-1KG
H2O	Autoclaved
Bacto-agar	BD, #214050
Ampicillin	Sigma, #A5354
DNA Maxi prep Kit	Sigma, #NA0400S / RT, 4°C
ACC1	Promega, ACC1 Rest. Enzyme #R6411 / -20°C
Buffer G	Promega, #R007A /-20°C
Sca1	Promega, Sca1 Rest. Enzyme #R6211 / -20°C
Buffer K	Promega, #R010A / -20°C
DNA Ladder	Invitrogen, #15628-019 / -20°C
DNA Dye	BostonBioProducts, Agarose gel loading Dye #BM-100G / 4°C
Agarose	Bio-Rad, #162-0102

3. Materials and Methods

<u>Name</u>	<u>Manufacturer/specifications</u>
Ethidium Bromide	Gibco, #15585-011 / RT
PCR Tubes	Eppendorf
Electrotransfection	Bio-Rad Micropulser
Centrifuges	Sorvall RC5C plus Superspeed Beckmann Coulter Allegra 6 Beckmann Coulter Microfuge 18
Photometers	Perkin-Elmer Lambda 3 UV/VIS Spectrophotometer Amersham Biosciences Gene Quant pro
Shaker	LLI Lab-line Incubator-Shaker
Microwave	Panasonic Inverter
Electrophoresis box	OWL Scientific Plastics, OSP-105
UV Images	Syngene Bio Imaging GENE FLASH
<u>Lipofection</u>	
Metafectene	Biontix, #T020-1.0 / 4°C
pOG44 Plasmid	Invitrogen
<u>Drugs and treatments</u>	
Hydroxyurea	Sigma, #H8627-1G /4°C
Thymidine	Sigma, # T1899-5G
Caffeine	Sigma, # C0750-5G
Weigh	Denver Instrument Company TL104 Fisherbrand Weighing Paper
Syringes	BD, 10ml Syringe
Filters	Fisherbrand 25mm Syringe

3. Materials and Methods

<u>Name</u>	<u>Manufacturer/specifications</u>
<u>Stainings</u>	
Sucrose 99%	Sigma, #S0398-500G / RT
PFA 4% (paraformaldehyde)	Boston Bioproducts, #BM154 / -20°C
BSA (bovine serum albumine)	Sigma, #A 9647-50G / -20°C
BGS	Hyclone, #30541.03 / 4°C
Triton X-100	Sigma, #T-9 284 / RT
Hepes	Siena / 4°C
NaCl	Sigma, #S3014-500G / RT
KCL	Sigma, #P4505-500G / RT
Mounting Medium	Vector, Vecatshield #H-1000 / 4°C
DAPI	Sigma, #D9542-1MG / 4°C
Slides	BD, #354108 / RT
Covers	Fisher Scientific, #125485C / RT
Clear Nail polish	New York Nails / RT
Humidified chamber	Tupperware box with moist paper
Fluorescence Microscope	Olympus America BX51
Source of fluorescence	Olympus BX-URA2
Camera	Zeiss AxioCam MR
<u>Antibodies</u>	
Rad 51	
1. Rad51 rabbit PAb	Calbiocam, #D00024394 / -20°C
2. Chicken anti-Rabbit yH2AX	Invitrogen, Alexaflour 20 #54256A / 4°C
1. Mouse mAb to yH2AX	Abcam, #ab18311-100 / -20°C
2. Goat anti-Mouse	Invitrogen, Alexaflour488 #A1102 / 4°C

4. Results

4.1 Establishing isogenic H1299 cell clones with different p53 status

The p53 mutant cell lines H1299FRT zeo, H1299 FLP QS22,23 and H1299 FLP QS22,23 S15A were successfully generated via site-directed mutagenesis of p53-expression-plasmids and Flp-In System transfection into host cell line.

All cell lines were derived from H1299, a non small cell lung carcinoma cell line which harbors a partial deletion of p53 and is thus functionally p53 null.

Figure 6a shows the linear sequence chart of p53 protein including both introduced N-terminal mutations at Q22, W23 and at S-15 (as generated in the H1299 FLP QS22,23 S15A cell line, see below). The single mutated H1299 FLP QS 22,23 cell line was transfected with a Q22L,W23S mutant, transactivation-inactive plasmid. This mutation was introduced to minimize the effects of transactivation and downstream activities of p53, enabling observations of direct protein interactions only. H1299 FLP QS cells are expected to suppress HR via direct ATR/p53/Rad51 interaction, thus forming fewer detectable Rad51 foci as compared to the p53-null variant.

H1299 FLP QS22,23 S15A cells are provided with an additional S15A mutation and are supposed to indicate the loss of HR suppression through increased Rad51 foci formation. This enhanced HR should be due to interruption of the direct interaction of p53 and ATR.

H1299 FRT zeo cells contain only the FRT target site and therefore reflect HR activity in p53 null cells. For simplicity, the cell lines will be named H1299 QS (for H1299 FLP QS 22,23), H1299 S15A (for H1299 FLP QS 22,23 S15A) and H1299 FRT (for H1299 FRT zeo) in the following text.

Since transfection does not guarantee equal protein expression levels in cell lines, a western blot was performed to determine whether comparable amounts of p53 proteins are expressed in both cell lines (immunoblot was performed by Bianca Sirbu). Figure 6b demonstrates that the tested H1299 QS and H1299 S15A cell lines both show basal levels of p53 protein comparable to functional p53 protein levels in wt p53 MCF7 cells. Since already very low levels of p53 are known to successfully suppress HR, minor p53 expression level deviations in H1299 S15A cells are negligible.

4. Results

HR is a cell cycle-dependent DNA repair mechanism, preferably executed during S- and G2 phase. Exposure to thymidine (TdR) disturbs the stoichiometric use of all four nucleotides for DNA synthesis. Thymidine thus slows down replication or even arrests cells during S-Phase. In order to determine whether expression of p53 QS or p53 S15A differentially affects cell cycle progression in the presence of the S-phase inhibitor, a cell cycle analysis with and without thymidine treatment was performed (by Bianca Sirbu). Figure 6c shows the distribution of cells in the various phases. It confirms that thymidine accumulates cells in S-phase independently of the p53 form expressed.

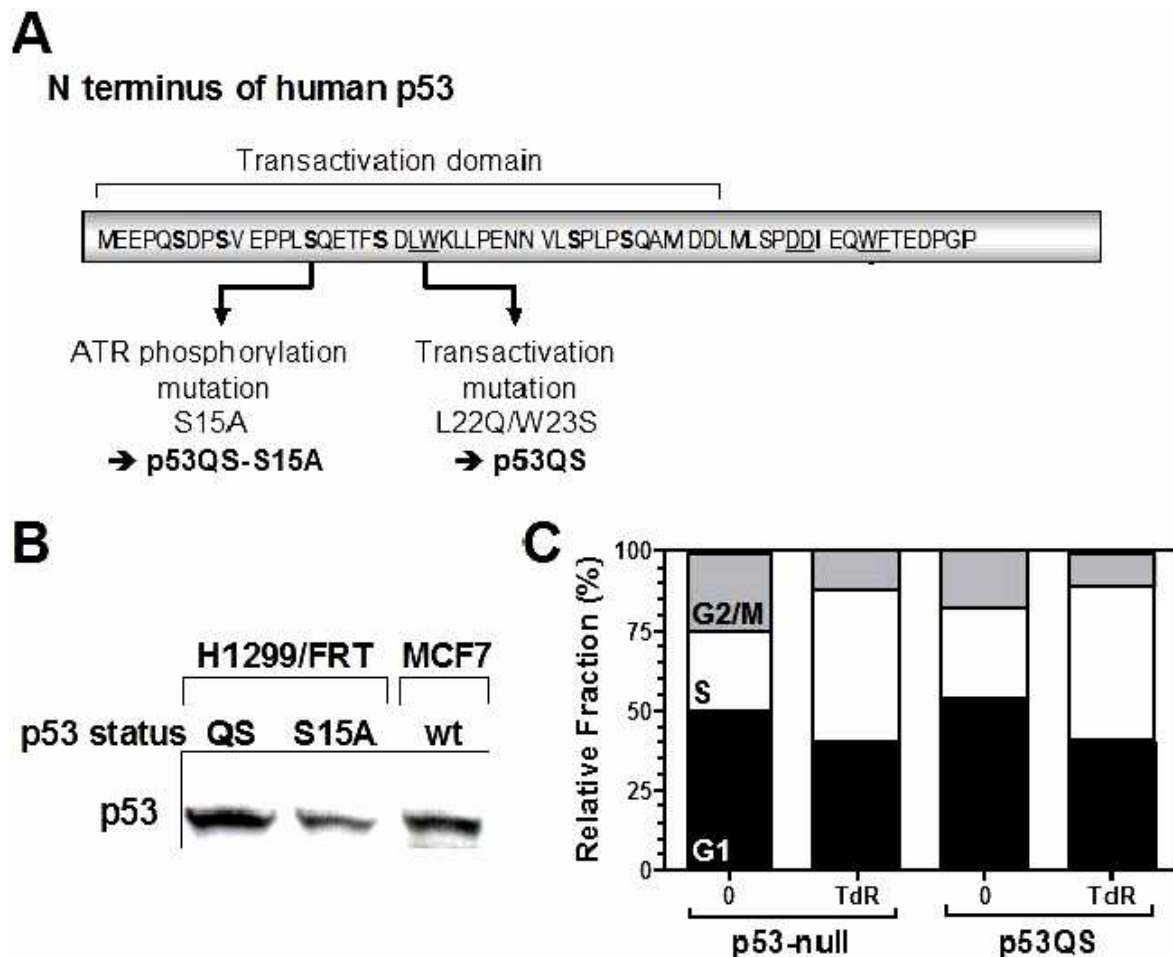


Figure 6: Location of the L22Q/W23S and the S15A mutations in the linear sequence chart of the N-terminus of p53 (a). Protein expression levels of p53 in the transfected H1299 cells compared to MCF7 wt cells (b). Cell cycle distribution of the p53 null and the transfected L22Q/W23S cell line, in untreated cells and after 24h 5mM TdR treatment (c) (With friendly permission of Henning Willers).

4. Results

4.2 Induction of Rad51 foci in response to replication fork stalling

Hydroxyurea ($C_1H_4N_2O_2$) and thymidine ($C_{10}H_{14}N_2O_5$) induce S-phase delay via inhibition of the ribonucleotide reductase and hence depletion of the dNTP pool (HU), or dysbalance of NTP usage (TdR). Despite different mechanisms both agents lead to replication fork stalling, resulting in elevated levels of HR. To decide whether HU or TdR should be used during the following experiments, cells were incubated with both drugs to compare the differences in Rad51 foci formation and appearance.

H1299 FRT and H1299 QS cell lines were plated overnight in 6 12-well chamber slides (4 chambers each slide, 3 slides each cell line) and treated with 5mM HU (2 slides) or 5mM TdR (2 slides) or left untreated (2 control slides). TdR appeared to be less toxic and generated larger, brighter and more distinct Rad51 foci (Fig. 7) as compared to HU. In the following experiments cell cycle inhibition was only performed via TdR.

In general, all cell lines, H1299 FRT, H1299 QS and H1299 S15A show different individual numbers of Rad51 foci prior to treatment (untreated control cells) and, according to their ability of suppressing HR, higher numbers of Rad51 foci after treatment.

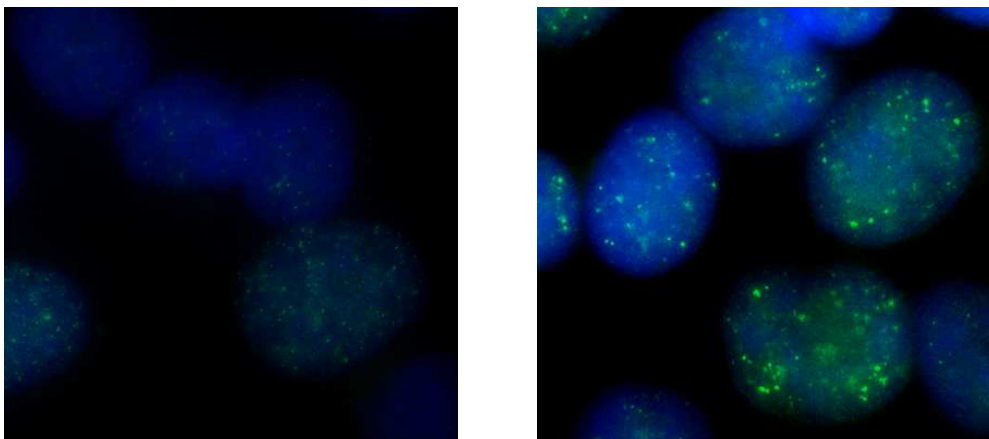


Figure 7: Overlay pictures of the nuclei of H1299 FRT cells immunostained for Rad51 foci following 5mM HU (left) and 5 mM TdR (right). The TdR treatment generated larger and brighter Rad51 foci than HU.

4. Results

Monitoring the formation of Rad51 foci is a widely used method to observe HR activity in mammalian cells. To compare HR levels in different cell lines, it was necessary to generate a quantitative criterion to discriminate effects of HR suppression or HR promotion. For this purpose, the individual numbers of Rad51 foci per cell were compared to determine a cut-off number to formally discriminate between HR-conducting or HR-negative cells. This cut-off had to be generated simultaneously for the cell lines to ensure the comparability of all consecutive results. Cells were incubated with 5mM TdR for 24h, fixed on slides and stained. Each slide was previously examined in DAPI staining to exclude apoptotic and fragmented nuclei from the counting process. 100 intact nuclei per well were counted. The individual numbers of foci per nucleus were then grouped in 1-4, 5-9, 10-14, 15-19, 20+ foci/nucleus. The numbers of cells that showed the respective numbers of foci were expressed as a percentage of the total number of cells, as demonstrated in figure 8.

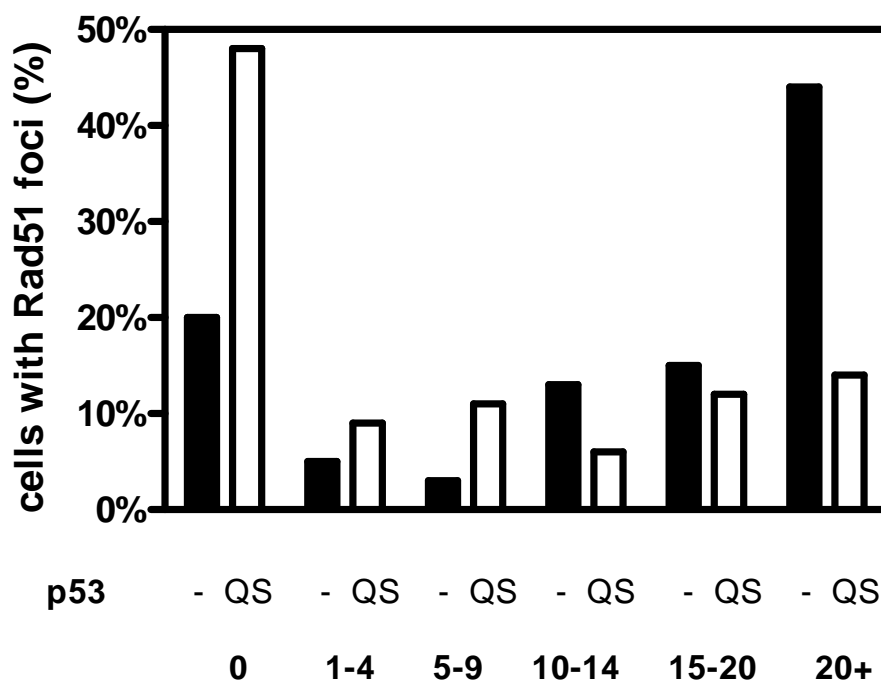


Figure 8: Distribution of Individual numbers of Rad51 foci per nucleus, in p53 null and QS cells after 24h 5mM TdR treatment.

4. Results

Several cut-off values were calculated and compared, the best discriminating cut-off turned out to be 10 foci. Enhanced numbers of cells with ≥ 10 foci were therefore considered to reflect elevated levels of HR. This cut-off was carried on for all consecutive experiments and cell numbers were plotted as the percentage of cells with ≥ 10 Rad51 relative to the total number of cells.

4.3 The S-15 site of p53 is required for suppression of Rad51 foci formation

The accumulation of Rad51 at stalled replication forks was induced by TdR treatment with 5mM for 24h. Control cells were left untreated (Fig. 9).

The percentages of cells with ≥ 10 Rad51 foci were compared for untreated cells and after 24h 5mM TdR exposition. All three cell lines double their number of cells with ≥ 10 foci after TdR treatment (Fig. 10). Compared to both cell lines H1299 FRT and H1299 S15A, H1299 QS cells showed clearly lower numbers of cells with ≥ 10 foci for control and TdR treated cells.

Because all three cell lines conduct spontaneous HR and exhibit individual basic numbers of cells with ≥ 10 foci in untreated cells, the actual TdR-induced numbers were calculated by subtracting the number of untreated cells with ≥ 10 foci from the number of treated cells with ≥ 10 foci (Fig. 10 “induced”, most right 3 columns). H1299 QS cells also showed fewer cells with ≥ 10 foci induced by TdR compared to the controls H1299 FRT, clearly indicating a suppressive effect of p53QS upon Rad51 foci formation.

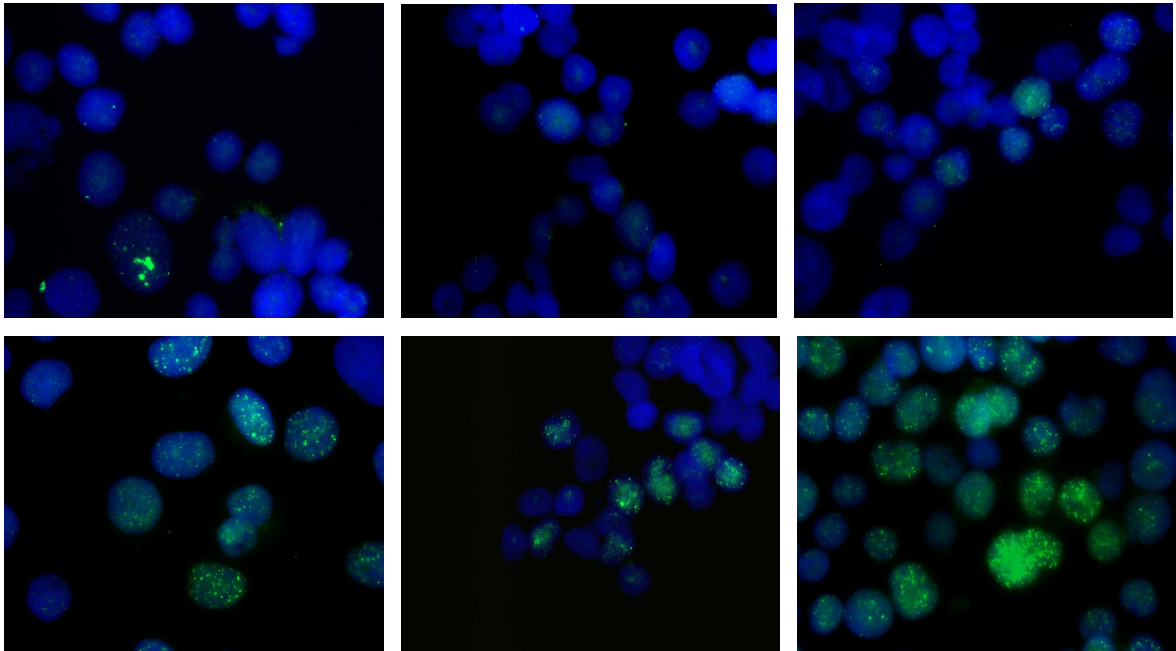


Figure 9: Pictures of Rad51 foci distribution in untreated (above) and after (below) 24h 5mM TdR treatment in H1299 FRT (left), H1299 QS (middle) and H1299 S15A (right) cells.

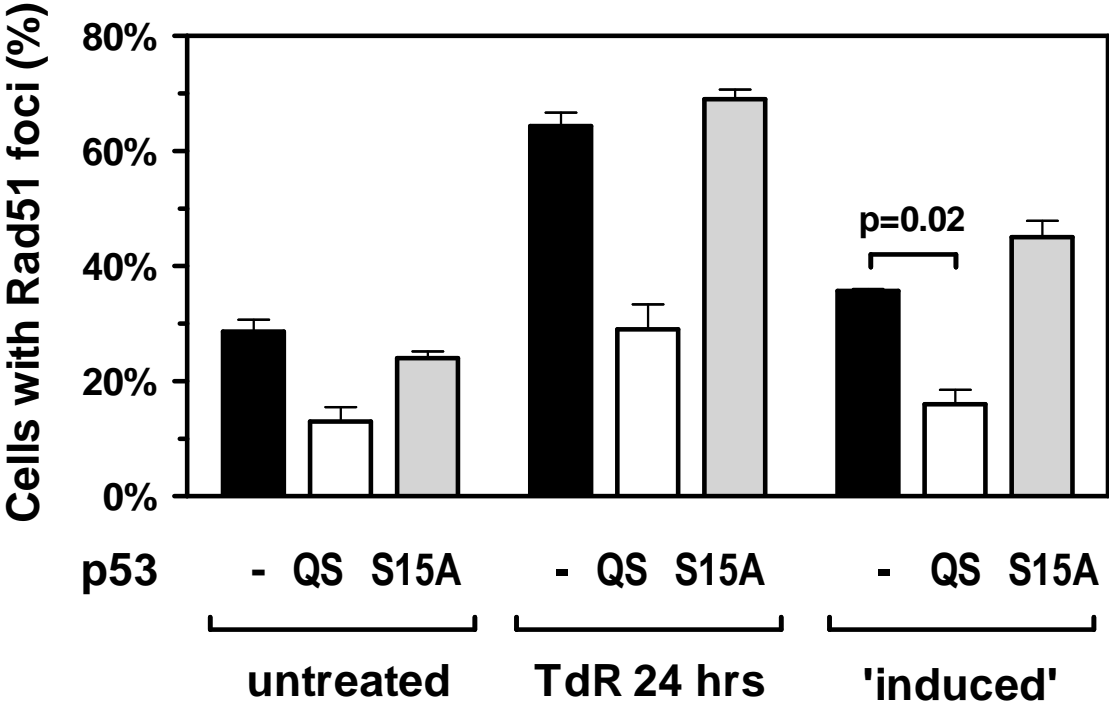


Figure 10: Distribution of cells with ≥ 10 Rad51 foci before and after 24h 5mM TdR and calculated columns for absolute, induced numbers of cells with ≥ 10 Rad51 foci.

4.4. Time courses of Rad51 foci formation

In order to observe whether the formation of Rad51 foci is time-dependent, its kinetics were monitored during the course of TdR treatment and after release from 24h TdR exposure by changing to regular medium. Cells were incubated with 5mM TdR as previously described and fixed and stained for Rad51 foci every 2h (Fig. 11). In all three cell lines, the majority of Rad51 foci formed within the first 8h during incubation with only a slight further increase thereafter. After 24h, all three cell lines approached their comparatively highest levels of nuclei with ≥ 10 Rad51 foci. All three cell lines showed similar kinetics of foci accumulation, however, in agreement with the previous results, the H1299 S15A cell line showed the highest level of Rad51 foci while H1299 QS experience the lowest percentage of cells ≥ 10 foci.

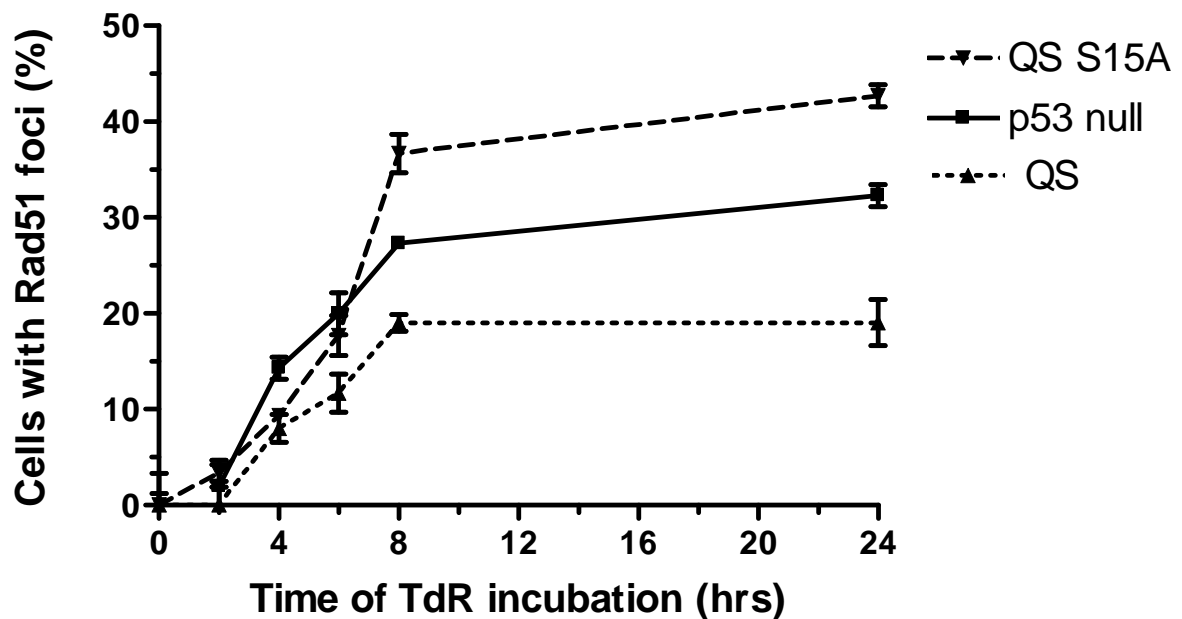


Figure 11: Kinetics of the cellcounts with ≥ 10 Rad51 foci during 5mM TdR incubation. The numbers of cells with spontaneous HR (untreated control cells) have been subtracted as described above, only numbers of cells with induced Rad51 foci are shown.

4. Results

After 24h of TdR incubation, the medium was substituted by TdR-free medium in order to release cells from replication fork stalling. All three cell lines showed a further strong increase in Rad51 foci formation (additional 10%), suggesting that after replication resumed, additional HR might be initiated to repair or restart the remaining stalled forks (Fig. 12). Subsequently the levels of Rad51 foci declined within the next 8h indicating a continuous slow repair process.

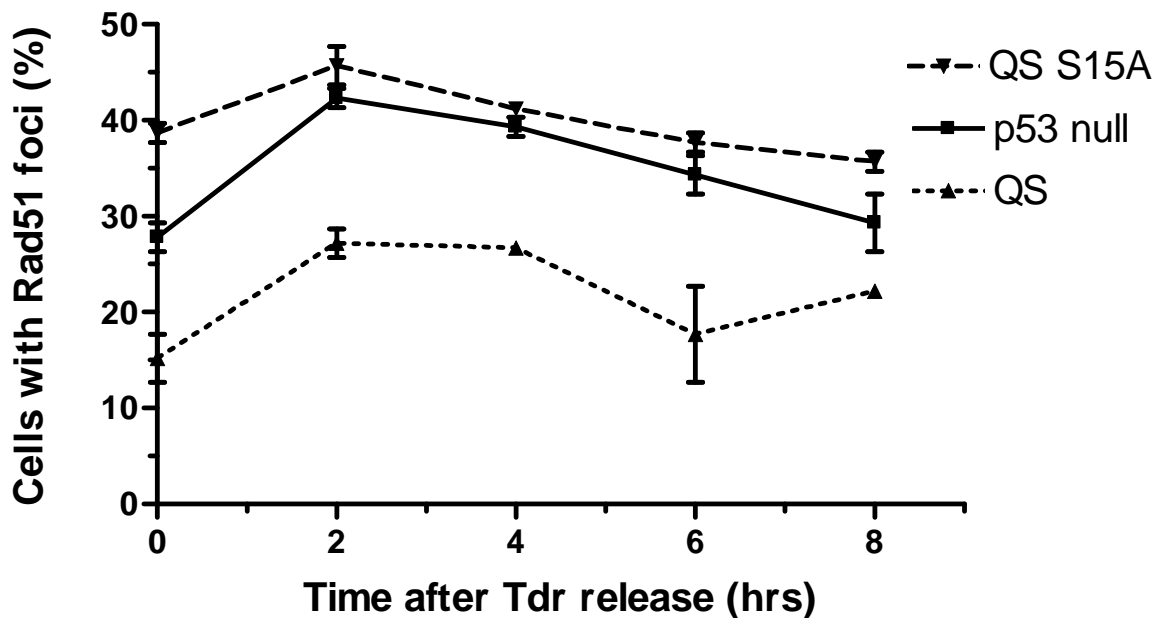


Figure 12: Kinetics of the cellcounts with ≥ 10 Rad51 foci after release from replication fork stalling and medium exchange. The numbers of cells with induced Rad51 foci are shown.

To determine whether the additional increase in Rad51 corresponded to plain DNA damage, cells were immunostained for γ -H2AX foci after TdR treatment (examples in Fig. 13). In order to investigate overall γ H2AX foci formation, cells were X-irradiated with 2Gy which uniformly lead to positive foci within 30min in all cells. After a 24h exposure to 5mM TdR, medium was replaced by regular medium and the level of γ H2AX foci was monitored every 2h.

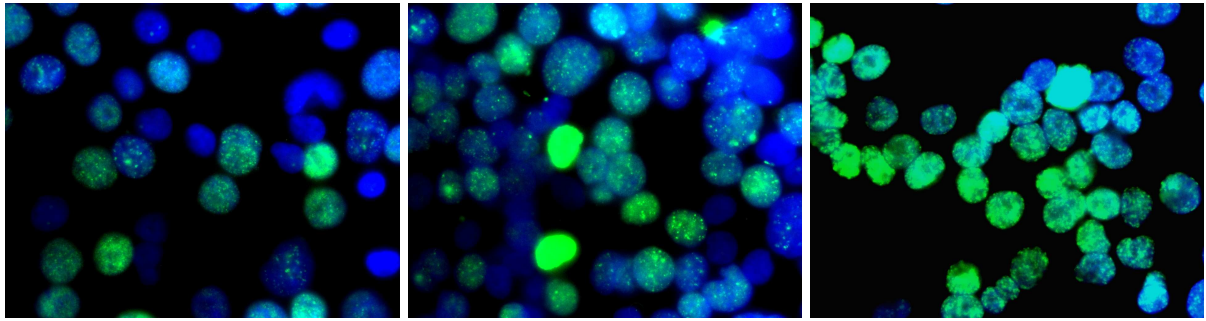


Figure 13: Pictures of γ -H2AX foci in H1299 S15A cells after 24h of TdR treatment(left), 2h after release (middle) and after IR.

Figure 14 demonstrates the induced percentage of cells with ≥ 10 γ -H2AX foci after 24h 5mM TdR, and 2h and 6h after release. 2h after removal of TdR the number of cells with ≥ 10 foci slightly increased and subsequently decreased again. The relative kinetics of γ H2AX and Rad51 foci are fairly parallel suggesting that both reflect at least partly the identical biological process, namely the formation and repair of DNA strand breaks. Notably, the number of cells with newly generated γ -H2AX foci after 24h TdR treatment was relatively low, indicating rather low rates of DNA strand break generation after TdR treatment. However the consistently higher number of Rad51 foci may reflect an additional effect purely associated with HR (see discussion).

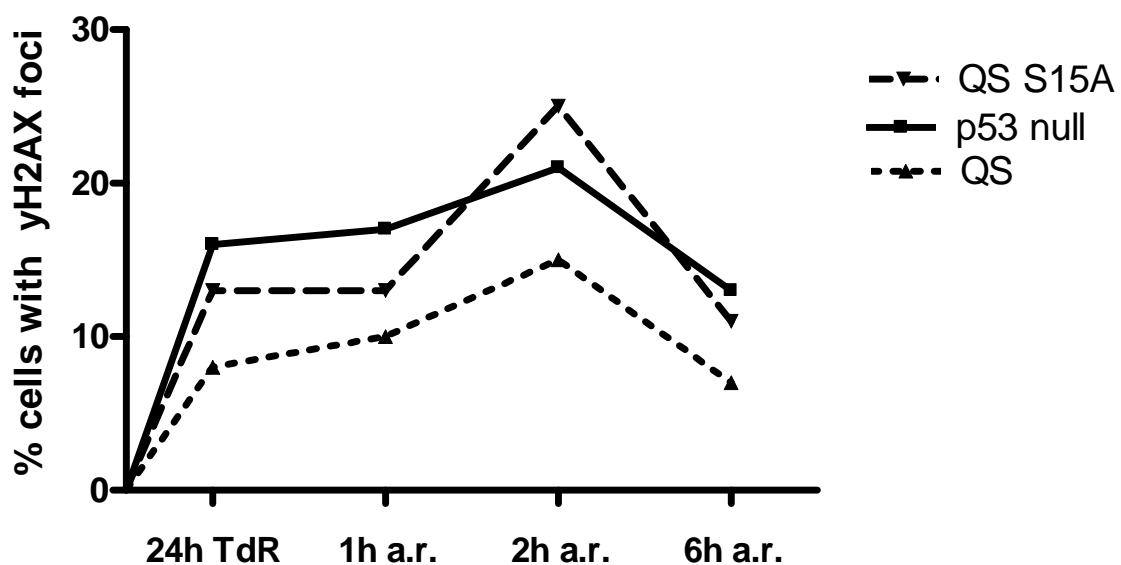


Figure 14: Kinetics of the cellcounts with ≥ 10 γ -H2AX foci after release from replication fork stalling. Only induced numbers of cells with γ -H2AX foci are shown.

4. Results

4.5 Differential effect of Caffeine upon induction and inhibition of Rad51 foci formation

Caffeine (Cf) is a strong inhibitor for both ATM and ATR kinase (in vitro IC_{50} for ATM=0.2mM, IC_{50} for ATR=1.1mM (115)). Because site-specific S-15 phosphorylation of p53 is an ATR/ATM dependent process, inhibition of the kinases via Cf was studied to further investigate the role of S-15 phosphorylation in HR. Cells were treated for 24h with different caffeine concentrations (0.4mM, 2mM, 5mM) (examples shown in Fig. 15).

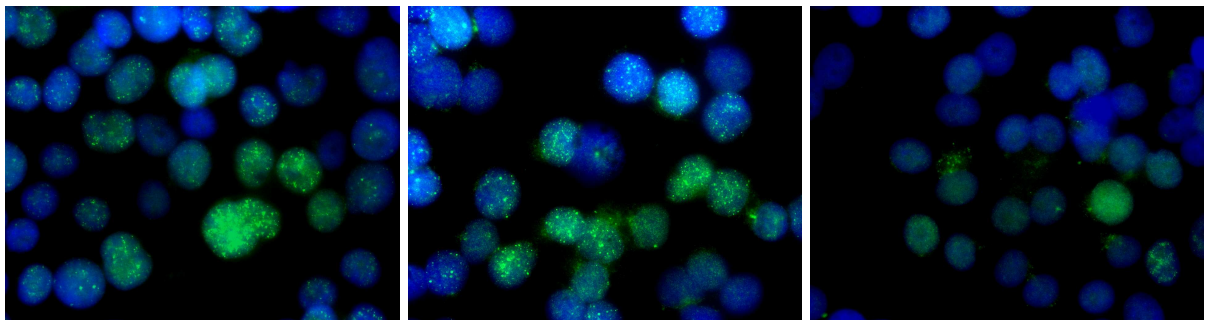


Figure 15: Rad51 foci in H1299 S15A cells after 24h TdR (left), 0.4mM Cf (middle) and 5mM Cf treatment.

Treatment with 0.4mM Cf alone increased the number of cells with ≥ 10 Rad51 foci by 2-folds while 2mM Cf did not induce foci exceeding the control levels. Treatment with 5mM Cf strongly suppressed the Rad51 foci formation to numbers even far below the levels in untreated control cells (Fig. 16).

The increase in cells with ≥ 10 Rad51 foci at lower Cf concentrations and the strong suppression of Rad51 foci formation at concentrations of 5mM Cf suggest a concentration-dependent inhibition of HR, presumably through suppression of ATR and ATM kinases at lower Cf concentrations, as well as through ATR/ATM/p53 independent suppressive effects of Cf on HR and cell cycle checkpoint signaling at higher concentrations.

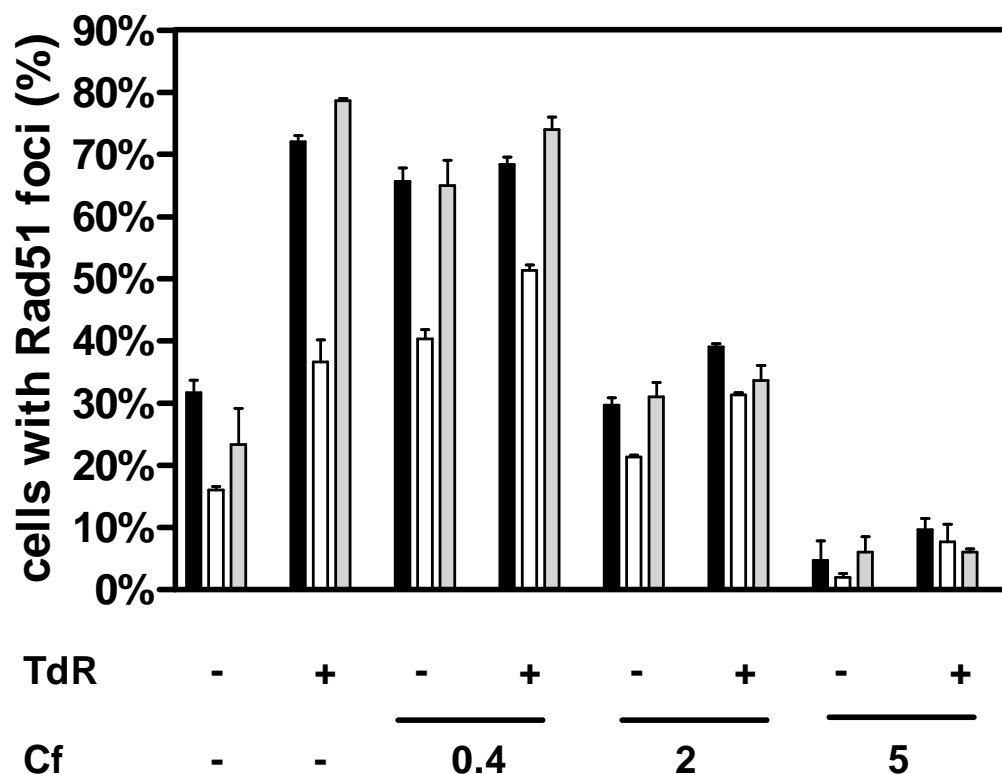


Figure 16: Distribution of numbers of cells with ≥ 10 Rad51 foci in untreated cells, after 24h 5mM TdR treatment and after treatment with different Cf concentrations. To avoid negative columns for the cells treated with 5mM Cf and for better comparison, the graph shows plain numbers of cells with ≥ 10 Rad51 foci, rather than the previously used induced numbers of cells with ≥ 10 Rad51 foci. The numbers for untreated control cells are shown in the 3 most left columns.

After simultaneous TdR (5mM) and Cf co-treatment at each concentration, all strains show a slight increase in numbers of cells with ≥ 10 Rad51 foci compared to controls (Fig.16). However, this increase was far below the level achieved by 5mM TdR alone, indicating a limit to levels of cells with ≥ 10 Rad51 foci in the observed population or a possibly suppressive effect of Cf on TdR metabolism.

The HR suppressing effect of H1299 QS cells compared to H1299 FRT and H1299 S-15A cells abrogates with increasing Cf concentration, indicating an additional, partly p53- independent effect of ATM/ATR on HR downregulation.

5. Discussion

Homologous recombination is one of the cells' most important DNA repair pathways. It enables error-free correction of DNA damage of various types and is therefore highly beneficial for cell survival as well as for the prevention of malignant transformation into cancer cells.

Being one of the most elaborate repair mechanisms, HR is regulated via promotion and downregulation by a number of highly orchestrated proteins and kinases, all of which contribute to maintain well-balanced levels of HR following damage exposure. This balance is crucial for cell viability, since elevated levels of HR during S-phase have been proposed to increase genomic instability in cells, resulting in mutations and aberrations of genomic material (67,159). Excessive HR leading to deleterious or missense recombination has been linked to carcinogenesis (5,117), and particularly proliferating cells have been shown to be highly prone to accumulation of HR induced DNA deletions (47,101). Considering the potential damage caused by excessive HR, the downregulation mechanisms of HR have been proposed to be an important additional system for the cell to prevent carcinogenesis.

This study was performed in order to observe the interactions between the tumoursuppressor protein p53 and the DNA damage response kinase ATR following replicative stress. It was investigated whether phosphorylation of p53 at the N-terminal serine residue S-15 via ATR is required for HR downregulation. A NSCLC H1299 cell system, consisting of one p53 null cell line, one transactivation-inactive cell line and one transactivation-inactive and S-15A mutant cell line, was used to monitor HR levels in the different experimental layouts. Replicative stress was induced via replication fork stalling through Thymidine, which is thought to mainly activate ATR as the principal damage response kinase following replication fork stalling. Taken together, the described study layout was constructed to observe differences in HR levels in the three cell strains, depending on their respective abilities to suppress HR via ATR-p53 interactions.

5. Discussion

5.1 transactivation-inactive H1299 L22Q,W23S p53 mutant cells retain their ability to suppress HR.

Suppression of HR via p53, as well as retaining of HR suppressive functions in transactivation-inactive p53 cells have been described and published for a wide range of different cell models (10,17,71,78,110,146,154), clearly showing a transactivation-independent effect for p53 on HR regulation. In this study, a plasmid containing a transactivation-inactive p53 mutant sequence has been introduced into the genome of H1299 NSCLC cells via Metafectene transfection. As a verification of the cell model used, Fig. 10 demonstrates the difference in HR levels in p53 null and QS cells before and after replication fork stalling treatment, showing that the QS cell line conducts significantly lower levels of HR than the p53 null cell line. These results, in consistence with previous studies, confirm that the transactivation-compromised H1299 QS cells are proficient to suppress HR effectively and independent of the cells basal HR levels.

5.2. The S-15 site of p53 is required for HR downregulation

The N-terminal S-15 site of p53 is the main target site for ATR/ATM- dependent phosphorylation in response to replicative stress. The damage response kinase preferably activated after exposure to replicative stress through replication fork stalling is ATR (see introduction) rather than ATM. Disrupted downstream signalling between ATR and p53 caused by impaired phosphorylation at S-15 is therefore thought to influence the cells habitual damage response and affect the kinetics of HR conduction. Figures 9 and 10 demonstrate that S15A mutant p53 cells that lack the ability of being phosphorylated by ATR, express significantly higher levels of HR after replication fork stalling compared to transactivation-inactive, but phosphorylation proficient QS cells. Consistent with data from previous publications (102), these findings show that phosphorylation of the S-15 site of p53 is required for successful HR downregulation.

Since the S-15 mutant cell line also carries the transactivation-inactivating L22Q, W23S mutation, the results are unlikely to be influenced by potential transcriptional activation or repression effects linked to S-15- phosphorylation.

A role for phospho-S-15 p53 in the regulation of HR has formerly been discussed in other publications which described colocalization and association of phospho-S-15 p53 with several HR key enzymes in response to replication fork stalling (33,103) In addition, Restle et al. (102) found the same HR suppressive effect of the phospho-S-15 site in a different cell model. Still, the exact mechanisms of the interactions between p53 and HR regulating enzymes remain subject of further investigations.

During the course of the experiment, HR levels in the S-15 mutant cell line were compared with the ones in p53 null cells (Fig. 10). Theoretically, the absence of p53 should result in comparatively higher levels of HR, due to the missing suppressive effect of p53. But despite containing an at least partially functional p53 protein, the number of cells conducting HR in the S15A cell line tended to exceed the levels of HR in p53 null cells in all experiments.

This could be explained in two ways: 1) S15 actively restricts pro-recombinogenic p53wt activity, or 2) The serine 15 to alanine mutation results in a gain of function mutation.

1) The S15 site of p53 naturally serves to downregulate excessive HR. On the other hand, p53 has been found to promote HR via a number of S-15 independent protein interactions and signalling pathways (see introduction). Phosphorylation at S-15 could therefore not only downregulate HR, but also counterbalance pro-recombinogenic, e.g. HR initiating and promoting properties of p53.

S-15 phosphorylation has further been shown to induce apoptosis in severely damaged cells (124). Disturbing the respective pathways could lead to an imbalance between induction of apoptosis versus DNA repair in the respective cells, resulting in elevated repair levels and less induction of apoptosis. Impairing S-15 phosphorylation via S15A mutation would therefore cause an unrestricted p53, whose unrestricted pro-recombinogenic properties as well as its imbalanced ratio between repair and apoptosis would contribute to higher levels of HR than in cells containing no p53 at all.

2) In addition, a gain-of-function mutation, in which a newly acquired mutation results in the promotion of oncogenic processes in cells rather than in a pure loss of suppression, can not be entirely excluded. In this case, S15A mutant p53 would cause excessive levels of HR in the respective cells and actively contribute to genomic destabilization via HR promotion.

5.3. HR levels increase after release from TdR induced replication fork stalling

Replication fork stalling can be achieved via a number of drugs, all of which stall the respective forks through different mechanisms. Thymidine was used in this study due to the advantage of specifically targeting the replication fork progression only and its lower toxicity compared to other stalling agents.

Figure 9a shows the time kinetics of the development of Rad51 foci following TdR treatment during a designated time period. Treatment with 5mM TdR resulted in an increase of HR levels in all cell lines during the first 5 to 8 hours followed by an only minor increment within the next 24 hours. Surprisingly, after medium exchange and release from the TdR block after 24h, a further significant increase in HR activity was detected in all three cell lines, reaching maximum HR levels within the first 2-3 hours after release. In order to investigate the increase of recombination events and its causes, a series of γ -H2AX immunostainings was conducted (Fig.13,14). Accumulation of γ -H2AX foci inside the nuclei is considered as an established indicator for the occurrence of DNA strand breaks (105,118). A rise in γ -H2AX foci after release of the TdR block could therefore reveal DNA damage or indicate other DNA strand break-inducing processes in the released cells, which would contribute to the rise in HR levels following TdR release. The number of cells with γ -H2AX foci slightly but significantly increased after a distinct time (approx. 2-4h) following TdR release, corresponding with the increase of Rad51 foci in cells conducting HR during the same time period. Contrarily to the levels of Rad51 foci, though, the formation of γ -H2AX foci remained at relatively low levels and did not show a similar high increase, raising the possibility that not all Rad51 foci reflect frank DSB but perhaps restart of stalled forks by several mechanisms (Fig. 17):

- 1) Replication fork stalling agents such as hydroxyurea and aphidicolin have been reported to be cytotoxic at certain higher concentrations, leading to unmanageable amounts of DSB and eventually cell death. Recent studies investigating the immediate effect of replication fork stalling describe little to no effect of TdR on DSB formation directly after treatment (18,75). Since the occurrence of DSB remains at low levels in the present results as well, the DSBs could therefore originate in spontaneous breakage or disruption of the single stranded ends, followed by the collapse of a small fraction of the stalled replication forks rather than being the result of an active damaging process. The collapsed forks would require the localised

phosphorylation of H2AX as well as activation of DNA repair pathways.

2) The occurrence of low numbers of intentionally introduced strand breaks could be the result of activation of Mus81, an endonuclease that is specialized on stalled replication forks as a substrate (8,42,54). Mus81 introduces SSBs and DSBs at the stalled forks, resulting in fork cleavage, repair, restart and replication progression. Depending on the type of break, the lesions are then detected by the respective DNA repair enzymes, inducing DNA repair and subsequent fork cleavage and progression (30). If a designated fraction of the stalled forks was repaired via Mus81, it would cause a small number of DNA strand breaks during this process.

Mechanisms to restart the stalled replication forks without introduction of strand breaks include the activation of new origin firing and of direct HR repair.

3) The long-term stalling of replication forks has been demonstrated to result in resumption of replication via new origin firing, which requires no formation of DSB at all (93). The remaining stalled fork intermediates situated in vicinity to the new origins can afterwards be restarted via several possible pathways, including NHEJ, SDSA or HR.

4) Previous observations have shown that replication fork restart is one of the specific purposes of HR and HR proteins (81,109,113). Examples for the used recombinant steps include template switching, Holliday junctions and the formation of chicken foot structures, all of which do not necessarily require a DSB or direct involvement of endonucleases at all (144). The increase in HR frequency after TdR release accompanied by relatively low levels of DSB in the present results could therefore be caused by an additional need of HR events, not only to repair, but also to restart the blocked replication forks at the time the block is resolved.

A similar distribution of Rad51 and γ -H2AX foci, though only monitored following release from long-term (24h+) fork stalling, has recently been described by Petermann et al. (93), who propose a plain collapse and inactivation of replication forks after long-term stalling, which is followed by rescue via HR, resulting in a strong increase in HR levels after release from stalling. Regarding the different co-existing mechanisms of restarting the forks after stalling, a simultaneous activation of these mechanisms, partly including the formation of strand breaks, could explain the comparably high rise in HR attended by a moderate rise in DSB.

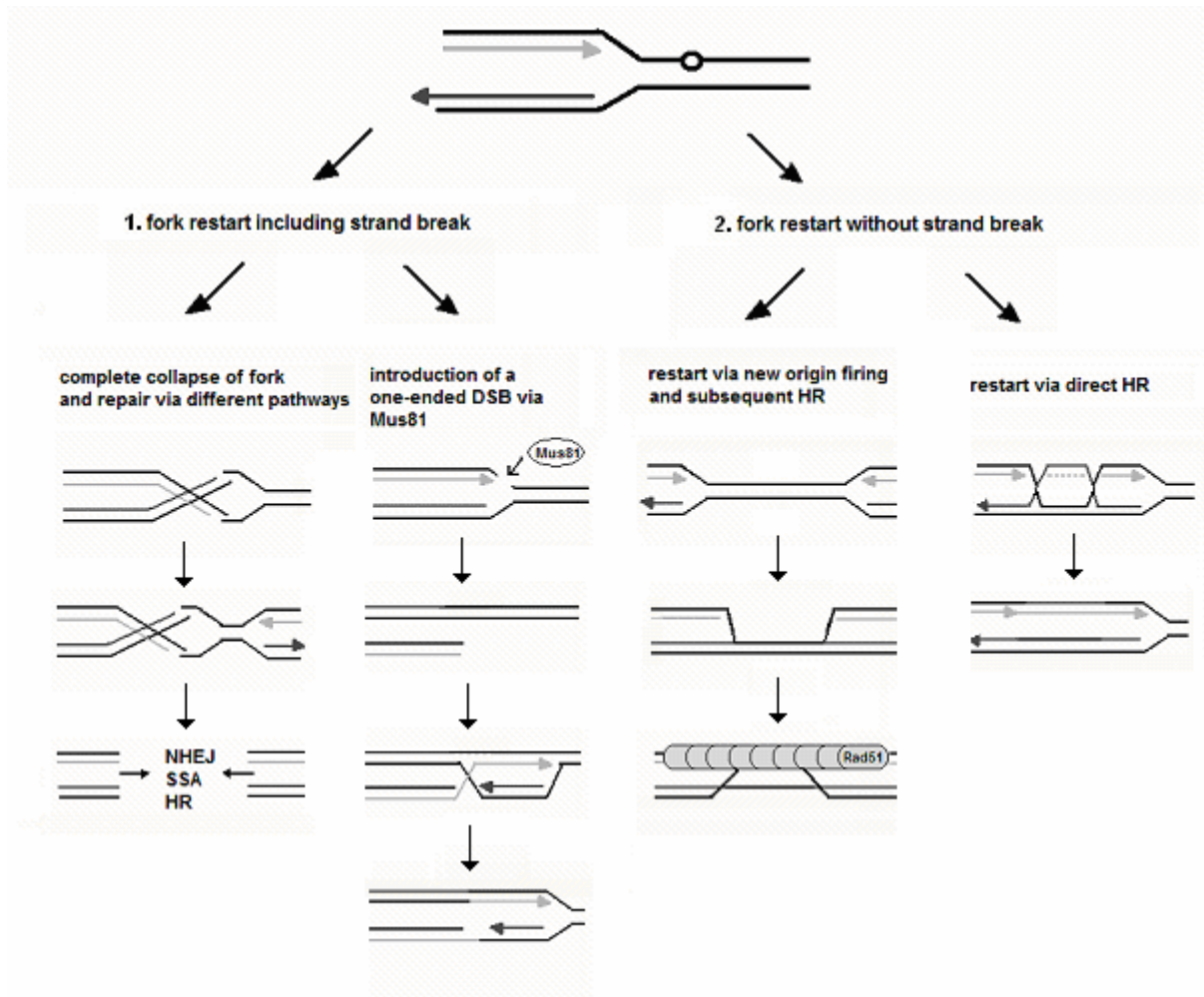


Figure 17: Examples of restart mechanisms of stalled replication forks. 1) Spontaneous breakage or introduction of strand breaks via Mus81, causing a rise in H2AX levels as well as in HR levels. .2) Fork restart without introduction of strand breaks via origin firing or direct HR, leading to elevated levels of HR only.

5.4 Low concentrations of caffeine lead to impaired HR suppression

The two best described effects of caffeine on cells are the attenuation of damage induced G2 and intra-S phase arrest and the delay of repair processes (107,138,139,155). Cells treated with caffeine show signalling defects similar to cells that harbor the autosomal-dominant disease Ataxia teleangiectasia, a disorder characterized by mutational deactivation of both ATM genes. Caffeine is therefore often used as an ATR/ATM inhibitor (115), in order to study the effects of the downstream effects of the kinases and their potential targets in DNA damage response and cell cycle regulation (99,108,140,151).

Low dose caffeine treatment (0.4mM) of the H1299 FRT (p53 null), H1299 QS and H1299 QS S15A cell lines resulted in a remarkable increase in HR frequency in all three cell lines (Fig. 16), indicating disruption of the HR downregulation signalling pathways. A similar effect of caffeine treatment has previously been found by Restle et al. (102) in a different cell model. The results of the present study, showing a simultaneous increase in HR frequency in the p53-null cell line along with the two other cell lines raise the question whether the process of HR downregulation is exclusively dependent on ATR-p53 interactions, or if the results may be caused by partially independent effects of ATR/ATM on HR suppression. Several mechanisms could be taken into account to explain these results.

- 1) ATR/ATM have p53-independent regulating effects on HR conduction and suppression. The stabilization of stalled replication forks is an important step during HR, in which the ATR/ATRIP complex has been shown to participate during replication inhibition (see introduction). This step has never been described to be dependent on interactions with p53. Inhibition of ATR/ATM via caffeine might lead to partly insufficient stabilization of the replication intermediates and therefore result in elevated numbers of collapsed forks, requiring higher levels of HR-activity in cells.

- 2) Caffeine has ATR/ATM-independent effects on HR. Caffeine is known to have various effects on the cells metabolism, which cannot entirely be based on ATR/ATM inhibition alone. Caffeine has been shown to influence DNA-PK (14), as well as several other enzymes participating in cell cycle regulation and DNA repair (139,140,151). Existing data support the notion that caffeine further affects downstream proteins of ATR, ATM and Chk1 (62), though explicit specifications of the involved pathways or enzymatic effects remain unclear at the present time.

Caffeine could therefore contribute to replication fork stalling, destabilization, or delay in the repair process itself by affecting the respective downstream proteins of ATR and ATM as well as directly influencing HR associated proteins such as Rad51, RPA or BRCA1/2.

3) Caffeine itself has been described to trigger oxidative stress, DNA damage and apoptosis. Caffeine treatment has repeatedly been shown to promote apoptosis in different cell models (39,52), though the specific affected or induced pathways remain unknown. In recent studies, Caffeine has as well been shown to directly induce oxidative stress (97) which could lead to DNA damage or replication fork stalling at the respective damaged sites and therefore promote increased or prolonged HR activity in response.

As a further result, the co-treatment with caffeine and the replication fork stalling agent TdR only slightly increased HR frequency compared to single TdR treatment. Since all cells of the respected caffeine-treated cell population situated in S-phase already conduct HR, these numbers cannot be increased by additional TdR treatment.

5.5 High concentrations of caffeine lead to complete inhibition of Rad51 foci formation and HR

Caffeine treatment at intermediate concentrations of about 2mM had a rather small effect on HR frequency in all cell lines (Fig. 16). HR levels hardly differed from the ones in untreated cells. Therefore, no promoting or suppressive effect of caffeine could be determined. An increase of the numbers of Rad51 foci, as observed after low concentrations, has not been found. Similar to the results shown after low-dose caffeine treatment, co-exposure of TdR and caffeine did not induce any significant increase in HR frequency in any of the cell lines. In this case however, the underlying cause cannot be explained by the maximum number of cells already conducting HR. Caffeine has been found to disrupt checkpoint-mediated cell cycle delay and to cause overriding of the G2 and S checkpoints at rising concentrations starting from 1mM (99,108,115,151), therefore after 2mM, more cells have rapidly traversed the S-phase and are thus not undergoing replication stalling, fork repair and restart at the

time of observation, eventually resulting in reduced levels of HR after Cf/TdR co-treatment.

In addition, rising concentrations of Caffeine have been shown to delay and to slow down incorporation of ^3H -Thymidine (62), this effect could probably result in incomplete or disturbed replication fork stalling in the present study and impair replication fork stalling and elevation of HR levels.

Treatment with 5mM of caffeine completely abrogated conduction of HR. Fig.16 (last 6 rows) shows HR frequencies in all three cell lines, after 24h of 5mM Cf treatment. All cell lines exhibit lower levels of HR than the ones found in untreated control cells, indicating a suppression of even spontaneous Rad51 formation. Further, the formation of Rad51 foci inside the single nuclei, regarding even visibly small foci, or foci numbers below the cut off line of ≥ 10 , which were not taken into account by the used counting method, were completely abrogated as well (Fig. 15). This complete suppression was also observed upon simultaneous TdR treatment (Fig.16).

The strong suppression of Rad51 foci formation could be explained by 1) the inhibition of ATR and ATM kinases and the subsequent missing activation of cell cycle checkpoints, as well as by 2) their function as damage response kinases, phosphorylating DNA lesion recognition and repair initiation proteins such as RPA or H2AX.

1) The striking effects of Caffeine upon Rad51 foci formation and the complete absence of Rad51 foci found in this study correspond to recent findings of Nabieh et al. (84), who hypothesized that treatment with caffeine leads to inhibition of ATR/ATM and other cell cycle control proteins. These cells override the respective cell cycle checkpoints and enter the state of mitosis prematurely, during which Rad51 foci have been shown to be completely absent (84,139).

2) Caffeine has further been found to inhibit DNA damage response through disrupting phosphorylation of RPA via DNA-PK and ATM (14,15) at Thr and Ser sites of the 32kDa subunit. Though little is known about the specific function of these sites, RPA itself is crucial for the stabilization of replication forks and interactions with Rad51 and its co-mediators, the inhibition of this process would also be a considerable explanation for the absence of Rad51 foci.

5. Discussion

Another protein involved in HR initiation is H2AX, which is phosphorylated at serine residue S139 shortly after the occurrence of DSB (105). This phosphorylation is conducted via co-existing pathways mainly by ATM, ATR and DNA-PK and leads to formation of γ -H2AX foci at the sites of the respective DSB (21,105,118). In case of dysfunction or inhibition of one of the kinases, H2AX phosphorylation is taken over and conducted by the remaining kinase (142,23). Several HR initiating proteins have been found to colocalize and associate with γ -H2AX, such as Mre11, Rad51 and Brca1 (91,40). Although the presence of γ -H2AX foci is not a crucial condition for successful conduction of HR (22), a stabilizing and supportive role for γ -H2AX at sites of DSB has been observed (23,96). Further, H2AX has been described to facilitate initiation of Rad51 foci formation, showing delayed Rad51 foci formation in H2AX mutant cells (127). A complete inactivation of both of the kinases responsible for H2AX phosphorylation could therefore result in the absence of γ -H2AX foci and consecutively disturbed or delayed initiation of HR.

The contrast between the strong increase in HR levels in all cell strains after 0.4mM and the complete absence of HR after 5mM of caffeine treatment has to be further investigated, but could potentially be the result of a consecutive inhibition of the two kinases ATR and ATM at different concentrations of Cf. In vitro IC_{50} have previously been described to be 0.2mM for ATM and 1.1mM for ATR (115). Accordingly, 0.4 mM may inhibit only ATM, resulting in replication fork destabilization and increased formation of strand breaks, which will require elevated HR levels to repair and reestablish the replication forks, while higher concentrations in addition abrogate ATR signaling which is required for Rad51 recruitment and HR during S-phase, leading to low levels of HR in all cells. This model would be consistent with the co-existing pathways of dual H2AX phosphorylation through ATR and ATM. The inhibition of ATM would therefore result in a counterbalanced phosphorylation of H2AX via ATR, still retaining successful initiation of HR. Inhibition of both kinases would completely abrogate H2AX phosphorylation, resulting in disturbed Rad51 foci formation and delayed association of repair proteins.

5. Discussion

While ATR has been characterized as the major damage response kinase for replication fork stalling, caffeine inhibits ATM as well as ATR, therefore an additional effect of ATM inhibition on the results of this study can not be excluded. Regarding the similar and often cooperating activity patterns of ATR and ATM in cell cycle control and DNA damage signaling, the step of p53 S15A phosphorylation in HR suppression could very likely be carried out by both of the kinases, depending on the type of DNA damage.

The original aim of this study was to investigate the effects of ATR-mediated S15A phosphorylation of p53 upon HR. The established results clearly indicate a significant effect of the S15A mutation on HR suppression, demonstrated by the elevated levels of Rad51 foci in the respective cell line.

Further, the effects of the inhibition of ATR/ATM and the identical kinetic reaction in all three cell lines indicate an additional, p53-independent involvement of ATR/ATM in HR regulation as well, questioning whether the site-specific phosphorylation of p53 is an essential step for HR downregulation.

Taken together, p53 S15 phosphorylation has been shown to generate a significant difference in HR levels in the respective cell lines. In combination with the p53-independent effects of ATR/AM on HR downregulation, the phosphorylation could therefore serve as an important additional control pathway to prevent excessive HR and contribute to the overall maintenance of genomic stability of the cell.

6. Summary

The purpose of this study was to investigate the influence of ATR-mediated phosphorylation of p53 at S15 on Homologous recombination.

Since DNA repair pathways, enzymes and their up- and downregulation play an important role in cancer genesis and moreover attain increasing importance in cancer treatment and therapy, it is crucial to gain further knowledge of the exact molecular mechanisms that promote or suppress the respective repair pathways.

In this study, Rad51 foci formation were employed as indicator of recombination activity. HR levels of three mutant cell strains were compared to determine whether ATR-mediated phosphorylation of p53 at S15 results in HR suppression. The cell system used for the experimental layout has been derived from the NSCLC H1299 cell line and consists of three variants of p53 mutants, one p53 null cell line, one p53 L22Q,W23S mutant and transactivation-inactive cell line and one L22Q,W23S S15A mutant cell line, which is transactivation-inactive and harbors an additional deficiency that prevents phosphorylation at S15 via ATR. Changes in HR levels or frequency in all cell lines were measured via quantification of Rad51 foci accumulation. Replicative stress was induced via replication fork stalling through thymidine, which is thought to mainly activate ATR as the principal damage response kinase following replication fork stalling. The inhibition of ATR was investigated via incubation with different concentrations of Caffeine.

This study was able to demonstrate that S-15 phosphorylation of p53 via ATR results in significant downregulation of HR. The presented results indicated a clearly impaired HR downregulation in the S15A mutated cell line compared to the other cell lines.

Further, the kinetics of the Rad51 foci formation during the course (2-24h) of TdR mediated replication fork stalling, as well as after release (24h +2h,4h,etc), were investigated and described. Despite the known effect of a rise in HR levels in all three cell strains during 24h of TdR treatment, the time course revealed an additional strong increase in HR activity similar in all three cell strains after release from 24h replication fork stalling. In order to study whether DNA damage has been cause of Rad51 accumulation, the formation of γ -H2AX foci during the same time period was studied. It was observed that the Rad51 foci accumulation was accompanied by a

simultaneous, though not equally strong rise in levels of γ -H2AX foci, due to several presumably corresponding replication fork restart pathways.

The inhibition of ATR/ATM through incubation with different Caffeine concentrations resulted in an initial strong increase in HR levels at 0.4mM Cf, followed by intermediate HR levels at 2mM, and a complete inhibition of HR at 5mM Cf, indicating additional, p53-independent regulatory activities of ATR/ATM upon HR downregulation.

Therefore, ATR-dependent phosphorylation of p53 at S-15 could serve as one of several coexisting downregulation mechanisms to suppress excessive HR and to reduce the risk of malignant transformation. In regard to recent developments in cancer therapy that target specific mutations in DNA repair pathways such as HR, it is highly important to gain thorough knowledge of the respective pathways and to identify potential target proteins, in order to provide individualized therapies suited for the underlying specific genomic alterations.

7. Literature

1. Abraham, R. T.: Cell cycle checkpoint signaling through the ATM and ATR kinases. *Genes Dev.* 2001; 15: 2177–2196.
2. Achanta, G. and Huang, P.: Role of p53 in sensing oxidative DNA damage in response to reactive oxygen species generating agents. *Cancer Res.* 2004; 64: 6233–6239
3. Andreassen P.R., D'Andrea A.D., Taniguchi T.: ATR couples FANCD2 monoubiquitination to the DNA-damage response. *Genes Dev.* 2004;18: 1958–63
4. Appella E., and Anderson C.W.: Post-translational modifications and activation of p53 by genotoxic stresses. *Eur J Biochem* 2001; 268: 2764-2772
5. Aubrecht J., Rugo R., and Schiestl R. H.: Carcinogens induce intrachromosomal recombination in human cells. *Carcinogenesis* 1995; 16, 2841–2846
6. Bakkenist, C. J., and M. B. Kastan.: DNA damage activates ATM through intermolecular autophosphorylation and dimer dissociation. *Nature* 2003; 421:499-506
7. Balakin G., Selivanova G., Yakoleva T., Kieseleva E., Kashuba E., Magnusson K.P., Szekely L., Klein G., Terenius L., Wiman K. G.: p53 binds single stranded DNA ends through the C-terminal domain and internal DNA segments via the middle. *Nucleic Acids Res* 1995, 23: 362-69
8. Bastin-Shanower S.A., Fricke W.M., Mullen J.R., Brill S.J.: The mechanism of Mus81-Mms4 cleavage site selection distinguishes it from the homologous endonuclease Rad1-Rad10. *Mol. Cell Biol.* 2003; 23: 3487–3496
9. Baumann P., Benson F.E., West S.C.: Human Rad51 protein promotes ATP-dependent homologous pairing and strand transfer reactions in vitro. *Cell* 1996; 87: 757–766
10. Bertrand P., Rouillard D., Boulet A., Levalois C., Soussi T. and Lopez B.S.: Increase of spontaneous intrachromosomal homologous recombination in mammalian cells expressing a mutant p53 protein. *Oncogene* 1997; 14: 1117-22
11. Bertrand P., Saintigny, Y. and Lopez, B.S.: p53's double life: transactivation independent repression of homologous recombination *Trends Gen* 2004; 20: 235-43
12. Bjursell G., and Reichard P.: Effects of thymidine on deoxyribonucleotide triphosphate pools and deoxyribonucleic acid synthesis in Chinese hamster ovary cells. *J. Biol. Chem.* 1973; 248. 3904-3909.
13. Bishop A. J. R., Hollander M. C., Kosaras B., Sidman R. L., Fornace Jr A. J., and Schiestl R. H.: Atm-, p53-, and Gadd45a-Deficient Mice Show an Increased Frequency of Homologous Recombination at Different Stages during Development *Cancer Res.* 2003; 63(17): 5335 - 5343

14. Block W.D., Merkle D., Meek K., and Lees-Miller S.P.: Selective inhibition of the DNA-dependent protein kinase (DNA-PK) by the radiosensitizing agent caffeine. *Nucleic Acids Res.* 2004; 32(6): 1967–1972
15. Block W.D., Yu Y., Lees-Miller S.P.: Phosphatidyl inositol 3-kinase-like serine/threonine protein kinases (PIKKs) are required for DNA damage-induced phosphorylation of the 32 kDa subunit of replication protein A at threonine 21. *Nucleic Acids Res.* 2004; 32(3):997-1005
16. Bode A.M. and Dong Z.: Post-translational modification of p53 in tumorigenesis. *Nat Rev Cancer*, 2004; 4(10): 793-805
17. Boehden G.S., Akyüz N., Roemer K., and Wiesmüller L. : p53 mutated in the transactivation domain retains regulatory functions in homology-directed double strand break repair. *Oncogene*, 2003; 22(26): 4111-4117
18. Bolderson E., Scorch J., Helleday T., Smythe C., and Meuth M.: ATM is required for the cellular response to thymidine induced replication fork stress. *Hum. Mol. Genet.* 2004; 13: 2937-2945
19. Buchhop S., Gibson MK., Wang X.Y., Wagner P., Stuerzbecher H.W. and Harris C.C.: F Interaction of p53 with the human Rad51 protein. *Nucleic Acids Res.* 1997; 25: 3868–3874
20. Bulavin D.V., Saito S., Hollander M.C., Sakaguchi K., Anderson C.W., Appella E., Fornace A.J.: Phosphorylation of human p53 by p38 kinase coordinates N terminal phosphorylation and apoptosis in response to UV radiation. *EMBO J* 1999; 18:6845-54
21. Burma S., Chen B.P., Murphy M., Kurimasa A., Chen D.J.: ATM phosphorylates histone H2AX in response to DNA double-strand breaks. *J. Biol. Chem.* 2001;276:42462–42467
22. Celeste A., Fernandez-Capetillo O., Kruhlak M.J., Pilch D.R., Staudt D.W., Lee A., Bonner R.F., Bonner W.M., Nussenzweig A.: Histone H2AX phosphorylation is dispensable for the initial recognition of DNA breaks. *Nat. Cell Biol.* 2003;5:675–679.
23. Chanoux R.A., Yin B., Urtishak K.A., Asare A., Bassing C.H., Brown E.J.: ATR and H2AX cooperate in maintaining genome stability under replication stress. *J Biol Chem* 2009;284:5994-6003
24. Chen, J., Lin, J. & Levine, A. J. Regulation of transcription functions of the p53 tumor suppressor by the mdm-2 oncogene. *Mol. Med.* 1995; 1: 142–152
25. Cimprich K.A., Cortez D.: ATR: an essential regulator of genome integrity. *Nat.Rev. Mol. Cell Biol.* 2008; 9: 616–627
26. Cortez, D., Guntuku S., Qin J., and Elledge S. J.: ATR and ATRIP: partners in checkpoint signaling. *Science* 2001; 294:1713–1716

27. Craig, A.L., Burch, L., Voitesek, B., Mikutowska, J., Thompson, A., Hupp, T.R., Novel phosphorylation sites of human tumor suppressor protein p53 at Ser20 and Thr18 that disrupt the binding of MDM2 protein are modified in human cancers. *Biochem J* 1999; 342: 133-141
28. Davies S.L., North P.S., Dart A., Lakin N.D., Hickson I.D.: Phosphorylation of the Bloom's syndrome helicase and its role in recovery from S-phase arrest. *Mol Cell Biol.* 2004; 24:1279–91
29. De Fromental C. and Soussi T.: TP53 tumor suppressor gene: a model for investigating human mutagenesis. *Genes Chromosomes Cancer* 1992; 4: 1–15
30. Doe C. L., Ahn J. S., Dixon J., and Whitby M. C.: 2002. Mus81-Eme1 and Rqh1 involvement in processing stalled and collapsed replication forks. *J. Biol. Chem.* 2002; 277: 32753-32759
31. Dohoney K M., Guillermin C., Whiteford C., Elbi C., Lambert P.F., Hager G.L., Brady J.N.: Phosphorylation of p53 at serine 37 is important for transcriptional activity and regulation in response to DNA damage. *Oncogene* 2004; 23:49-57
32. Dornan D., Hupp T.R.: Inhibition of p53-dependent transcription by BOX-1 phospho-peptide mimetics that bind to p300. *EMBO* 2001; 2: 139-144
33. Dudenhoffer C., Rohaly G., Will K., Deppert W. and Wiesmueller L.: Dissociation of the recombination control and the sequence-specific transactivation function of p53. *Oncogene* 1999; 18: 5773–5784
34. Dudenhoffer C., Rohaly G., Will K., Deppert W., Wiesmuller L.: Specific mismatch recognition in heteroduplex intermediates by p53 suggests a role in fidelity control of homologous recombination. *Mol Cell Biol* 1998; 8: 5332-42
35. Durocher D., and Jackson S. P.: DNA-PK, ATM and ATR as sensors of DNA damage: variations on a theme? *Curr. Opin. Cell Biol.* 2001; 13: 225–231
36. Egger A.L., Inman R.B., Cox M.M.: The Rad51-dependent pairing of long DNA substrates is stabilized by replication protein A *J Biol Chem* 2002; 277:39280-39288
37. Ellison V., Stillman B.: Biochemical characterization of DNA damage checkpoint complexes: clamp loader and clamp complexes with specificity for 5' recessed DNA, *PLoS Biol.*2003; 1:E33
39. Fernandez M.J., Lopez A., Santa-Maria A.: Apoptosis induced by different doses of caffeine on Chinese hamster ovary cells. *J. Appl. Toxicol.* 2003;23:221–224
40. Fernandez-Capetillo O., Celeste A., Nussenzweig A.: Focusing on foci: H2AX and the recruitment of DNA-damage response factors. *Cell Cycle.* 2003;2:426–427.
41. Fong P.C., Boss D.S., Yap T.A., Tutt A., Wu P., Mergui-Roelvink M., Mortimer P., Swaisland H., Lau A., O'Connor M.J., Ashworth A., Carmichael J., Kaye S.B., Schellens J.H., de Bono J.S.: Inhibition of poly(ADP-ribose) polymerase in tumors from BRCA mutation carriers. *NEJM* 2009; 361(2):123–134

42. Fricke W.M., Bastin-Shanower S.A., Brill S.J.: Substrate specificity of the *Saccharomyces cerevisiae* Mus81-Mms4 endonuclease. *DNA Repair (Amsterdam)* 2005; 4: 243–25
43. Friedman P. N., Chen X., Bargonetti J. and Prives C.: The p53 protein is an unusually shaped tetramer that binds directly to DNA. *Proc. Natl Acad. Sci. USA* 1993; 90: 3319–332
44. Fritsche M., Haessler C., Brandner G. Induction of nuclear accumulation of the tumorsuppressor protein p53 by DNA-damaging agents. *Oncogene* 1993; 307-18
45. Fox R. M., Mynderse J. F. and Goulian. M.: Incorporation of deoxynucleotides into DNA by diethylaminoethyl-dextran-treated lymphocytes. *Biochemistry* 1977;16: 4470-4477.
46. Fuchs B., O'Connor D., Fallis L., Scheidtmann K.H., Lu X.: p53 phosphorylation mutants retain transcription activity. *Oncogene* 1995; 10: 789-793
47. Galli A. and Schiestl R. H.: Cell division transforms mutagenic lesions into recombinogenic lesions in yeast cells. *Mutat. Res.* 1999; 429: 13–26
48. Gatz S.A. and Wiesmueller L., p53 in recombination and repair, *Cell Death and Differentiation* 2006; 13: 1003–1016
49. Glover T.W., Arlt M.F., Casper A.M., Durkin S.G.: Mechanisms of common fragile site instability. *Hum. Mol.Genet.* 2005; 14 :(2) R197–R205
50. Gottifredi, V., Shieh, S. Y., Taya, Y., and Prives, C. p53 accumulates but is functionally impaired when DNA synthesis is blocked. *Proc. Natl. Acad. Sci. USA* 2001; 98: 1036-1041
51. Hainaut P., Hollstein, M.: p53 and human cancer: the first ten thousand mutations. *Adv Cancer Res* 2000; 77:81-137
52. He Z., Ma W.Y., Hashimoto T., Bode A.M., Yang C.S., Dong Z.: Induction of apoptosis by caffeine is mediated by the p53, Bax, and caspase 3 pathways. *Cancer Res.* 2003;63:4396–4401
53. Helleday T., Lo J., van Gent D.C., and Engelward B.P.: DNA double-strand break repair: from mechanistic understanding to cancer treatment. *DNA Repair (Amst)*, 2007; 6(7): 923-935
54. Heyer W.D., Ehmsen K.T., Solinger J.A.: Holliday junctions in the eukaryotic nucleus: resolution in sight? *Trends Biochem. Sci.* 2003; 28:548–557
55. Hollstein M., Sidransky D., Vogelstein B., and Harris, C.C: p53 mutations in human cancers. *Science* 1991; 253, 49–53
56. Holmberg C.I., Tran S.E., Eriksson J.E. and Sistonen L.: Multisite phosphorylation provides sophisticated regulation of transcription factors. *Trends Biochem Sci* 2002; 27: 619-27

57. Hyrien O.: Mechanisms and consequences of replication fork arrest. *Biochimie* 2000; 82: 5–17
58. Itoh T., O’Shea C. and Linn S.: Impaired regulation of tumor suppressor p53 caused by mutations in the xeroderma pigmentosum *DDB2* gene: mutual regulatory interactions between p48DDB2 and p53. *Mol. Cell Biol.* 2003; 23: 7540–7553
59. Janus F., Albrechtsen N., Dornreiter I., Wiesmüller L., Grosse F., Deppert W.: The dual role model for p53 in maintaining genomic integrity. *CMLS Cell Mol. Life Sci.* 1999; 55: 12-27
60. Jiang G., Sancar A.: Recruitment of DNA damage checkpoint proteins to damage in transcribed and nontranscribed sequences. *Mol. Cell Biol.* 2006; 26:39–49
61. Jiang M., Axe, T., Holgate, R., Rubbi, C.P., Okorokov, A.L., Mee, T. and Milner, J.: p53 binds the nuclear matrix in normal cells: binding involves the proline-rich domain of p53 and increases following genotoxic stress. *Oncogene* 2001; 20: 5449–5458
62. Kaufmann W.K., Heffernan T.P., Beaulieu L.M., Doherty S., Frank A.R., Zhou Y., Bryant M.F., Zhou T., Luche D.D., Nikolaishvili-Feinberg N., Simpson D.A., Cordeiro-Stone M.: Caffeine and human DNA metabolism: the magic and the mystery. *Mutat Res.* 2003; 532(1-2): 85-102
63. Kumagai A., Dunphy W.G.: Claspin, a novel protein required for the activation of Chk1 during a DNA replication checkpoint response in *Xenopus* egg extracts, *Mol. Cell* 2000; 6:839–849
64. Lane D.P.: p53, guardian of the genome. *Nature* 1992; 358: 15–16
65. Lazaro-Trueba I., Arias C., Silva A.: Double bolt regulation of Rad51 by p53: a role for transcriptional repression. *Cell Cycle* 2006; (10):1062-5
66. Lee S., Elenbaas B., Levine A., Griffith J.: p53 and its 14kDa C-terminal domain recognize primary DNA damage in the form of insertion/deletion mismatches. *Cell* 1995; 81: 1013-20
67. Lengauer C., Kinzler K. W., and Vogelstein B.: Genetic instabilities in human cancers. *Nature* 1998; 396: 643–649
68. Levine A.J., Momand J. and Finlay C.A.: The p53 tumour suppressor gene. *Nature* 1991; 351,453–456
69. Levine, A.J.: p53, the cellular gatekeeper for growth and division. *Cell* 1997; 88: 323–331
70. Li W., Kim S.M., Lee J., Dunphy W.G.: Absence of BLM leads to accumulation of chromosomal DNA breaks during both unperturbed and disrupted S phases. *J Cell Biol.* 2004;165: 801–12

71. Linke S.P., Sengupta S., Khabie N., Jeffries B.A., Buchhop S., Miska S., Henning W., Pedoux R., Wang X.W., Hofseth L.J., Yang Q., Garfield S.H., Sturzbecher H.W., Harris C.C.: p53 interacts with hRAD51 and hRAD54, and directly modulates homologous recombination. *Cancer Research*, 2003; 63(10): 2596-2605
72. Liu Q., Guntuku S., Cui X., Matsuoka S., Cortez D., Tamai K., Guangbin Luo G., Carattini-Rivera S., DeMayo F., Bradley A., Donehower L.A. and Elledge S.J.: Chk1 is an essential kinase that is regulated by Atr and required for the G2/M DNA damage checkpoint. *Genes Dev.* 2000; 14: 1448–1459
73. Liu, Y. & Kulesz-Martin, M.: p53 protein at the hub of cellular DNA damage response pathways through sequence specific and non-sequence-specific DNA binding. *Carcinogenesis* 2001; 6: 851-860
74. Lu X., Lozano G. and Donehower L.A.: Activities of wildtype and mutant p53 in suppression of homologous recombination as measured by a retroviral vector system. *Mut Res.* 2003; 522: 69–83
75. Lundin C., Erixon K., Arnaudeau C., Schultz N., Jenssen D., Meuth M., Helleday T.: Different roles for nonhomologous end joining and homologous recombination following replication arrest in mammalian cells. *Mol Cell Biol.* 2002; 16:5869-78
76. Majka J., Binz S.K., Wold M.S., Burgers P.M.: Replication protein A directs loading of the DNA damage checkpoint clamp to 5' DNA junctions *J. Biol. Chem.* 2006 ; 281:27855–27861 80
77. Marmorstein L.Y., Ouchi T. and Aaronson S.A.: The BRCA2 gene product functionally interacts with p53 and Rad51. *Proc. Natl. Acad. Sci. USA* 1998; 95: 13869–13874
78. Mekeel K.L., Tang W., Kachnic L.A., Luo C.M., DeFrank J.S. and Powell S.N.: Inactivation of p53 results in high rates of homologous recombination. *Oncogene* 1997; 14: 1847-57
79. Melnikova V. O., Santamaria A. B., Bolshakov S. V. and Ananthaswamy, H. N.: Mutant p53 is constitutively phosphorylated at Serine 15 in UV-induced mouse Skin tumors: involvement of ERK1/2 MAP kinase. *Oncogene* 2003; 22:5958–5966
80. Melo J., Toczyski D.: A unified view of the DNA-damage checkpoint, *Curr. Opin. Cell Biol.* 2002; 14: 237–245
81. Michel B., Grompone G., Flores M. J., and Bidnenko V.: Multiple pathways process stalled replication forks. *Proc. Natl. Acad. Sci. USA* 2004; 101:12783-12788
82. Midgley C. A., Lane D. P.: p53 protein stability in tumor cells is not determined by mutation but is dependent on MDM2 binding. *Oncogene.* 1997; 15: 1179-1889
83. Momand, J., Zambetti, G.P., Olson, D.C., George, D.L., and Levine, A.J.: The mdm-2 oncogene product forms a complex with the p53 protein and inhibits p53-mediated transactivation. *Cell* 1992; 69, 1237-1245

84. Nabieh A., Rajendra E., Su X., Jeyasekharan A.D., Mahen R., and Venkitaraman A.R.: The Carboxyl Terminus of Brca2 Links the Disassembly of Rad51 Complexes to Mitotic Entry. *Curr Biol.* 2009; 19(13): 1075–1085
85. Nelson W. G., Kastan M. B.: DNA strand breaks: the template alterations that trigger p53-dependent DNA damage response pathways. *Mol. and Cell. Biol.* 1994; 14, No3: 1815-1823
86. Nyberg K.A., Michelson R.J., Putnam C.W., Weinert T.A., Toward maintaining the genome: DNA damage and replication checkpoints. *Annu. Rev. Genet.* 2002; 36:617–656
87. Oda K., Arakawa H., Tanaka T., Matsuda K., Tanikawa C., Mori T., Nishimori H., Tamai K., Tokino T., Nakamura Y., Taya Y.: p53AIP1, a potential mediator of p53 dependent apoptosis, and its regulation by Ser-46-phosphorylated p53. *Cell* 2000; 102:849-62
88. Okorokov, A.L.: p53 in a crosstalk between DNA repair and cell cycle checkpoints. *Cell Cycle* 2003; 2: 233-5
89. Osman F., Whitby M.C.: Exploring the roles of Mus81-Eme1/Mms4 at perturbed replication forks DNA Repair 2007; 6:1004–1017
90. Paull T.T., Gellert M.: The 3' to 5' exonuclease activity of Mre11 facilitates repair of DNA double-strand breaks. *Mol. Cell* 1998; 1: 969–979
91. Paull T.T., Rogakou E.P., Yamazaki V., Kirchgessner C.U., Gellert M., Bonner W.M.: A critical role for histone H2AX in recruitment of repair factors to nuclear foci after DNA damage. *Curr Biol* 2000;10:886-895
92. Pavletich N.P., Chambers K.A., Pabo C.O.: The DNA-binding domain of p53 contains the four conserved regions and the major mutation hot spots. *Genes Dev.* 1993, 7: 2555-64
93. Petermann E., Orta M.L., Issaeva N, Schultz N., and Helleday T.: Hydroxyurea-Stalled Replication Forks Become Progressively Inactivated and Require Two Different RAD51-Mediated Pathways for Restart and Repair. *Mol Cell* 2010; 37: 492–502
94. Petukhova G., Stratton S., Sung P.: Catalysis of homologous DNA pairing by yeast Rad51 and Rad54 proteins, *Nature* 1998; 393: 91–94
95. Pichierri P, Rosselli F, Franchitto A. Werner's syndrome protein is phosphorylated in an ATR/ATM-dependent manner following replication arrest and DNA damage induced during the S phase of the cell cycle. *Oncogene.* 2003; 22:1491–500
96. Pilch D.R., Sedelnikova O.A., Redon C., Celeste A., Nussenzweig A., Bonner W.M.: Characteristics of gamma-H2AX foci at DNA double-strand breaks sites. *Biochem Cell Biol.* 2003; 81(3):123-9

97. Pin-Zhen L., Ching-Yu L., and Wen-Hsiung C.: Caffeine Induces Cell Death via Activation of Apoptotic Signal and Inactivation of Survival Signal in Human Osteoblasts. *Int J Mol Sci.* 2008 5: 698–718
98. Piskley S.M., Lane D.P.: The p53-mdm2 autoregulatory feedback loop: a paradigm for the regulation of growth control by p53? *Bioessays*, 1993; 15: 689-690
99. Powell S. N., DeFrank J. S., Connell P., Eogan M., Preffer F., Dombkowski D., Tang W., and Friend S.: Differential sensitivity of p53(-) and p53(+) cells to caffeine-induced radiosensitization and override of G2 delay. *Cancer Res.* 1995; 55: 1643–1648
100. Reed M., Woelker B., Wang P., Wang Y., Anderson M.E. and Tegtmeyer P.: The C-terminal domain of p53 recognizes DNA damaged by ionizing radiation. *Proc. Natl. Acad. Sci. USA* 1995; 92: 9455–9459
101. Reliene R, Bishop A.J.R. and Schiestl R.H.: Involvement of Homologous Recombination in Carcinogenesis. *Adv. Genet.* 2007; 58:67-87
102. Restle A., Färber M., Baumann C., Böhringer M., Scheidtmann K.H., Müller-Tidow C., and Wiesmüller L.: Dissecting the role of p53 phosphorylation in homologous recombination provides new clues for gain-of-function mutants *Nucleic Acids Res.* 2008; 36(16): 5362–5375
103. Restle, A., Janz, C., Wiesmueller, L.: Differences in the association of p53 phosphorylated on serine 15 and key enzymes of homologous recombination. *Oncogene* 2005; 24: 4380–4387
104. Roemer K., and Mueller Lantzsch, N.: p53 transactivation domain mutant Q22,S23 is impaired for repression of promoters and mediation of apoptosis. *Oncogene* 1996; 12: 2069-2079
105. Rogakou E.P., Pilch D.R., Orr A.H., Ivanova V.S., Bonner W.M.: DNA double-stranded breaks induce histone H2AX phosphorylation on serine 139. *J. Biol. Chem.* 1998;273:5858–5868
106. Romanova L.Y., Willers H., Blagosklonny M.V. and Powell S.N.: The interaction of p53 with replication protein A mediates suppression of homologous recombination. *Oncogene* 2004; 23: 9025–9033
107. Rowley R., Zorch M., Leeper D.B.: Effect of caffeine on radiation-induced mitotic delay: delayed expression of G2 arrest. *Radiat. Res.* 1984; 97:178–85
108. Russell K. J., Wiens L. W., Demers G. W., Galloway D. A., Plon S. E., and Groudine M.: Abrogation of the G2 checkpoint results in differential radiosensitization of G1 checkpoint-deficient and G1 checkpoint-competent cells. *Cancer Res.* 1995; 55: 1639–1642

109. Saintigny Y., Delacote F., Vares G., Petitot F., Lambert S., Averbeck D. and Lopez B. S: Characterization of homologous recombination induced by replication inhibition in mammalian cells. *EMBO J.* 2001; 20: 3861-3870
110. Saintigny Y., Rouillard D., Chaput B., Soussi T., and Lopez B. S.: Mutant p53 proteins stimulate spontaneous and radiation-induced intrachromosomal homologous recombination independently of the alteration of the transactivation activity and of the G1 checkpoint. *Oncogene* 1999; 18: 3553–3563
111. Saito S., Appella E., Anderson C. W., Seaman W. T., and Kenney S.: ATM mediates phosphorylation at multiple p53 sites, including Ser(46), in response to ionizing radiation. *J. Biol. Chem.* 2002; 277: 12491–12494
112. Sakaguchi K., Sakamoto H., Lewis M. S., Anderson C. W., Erickson J. W., Appella E., Xie D.: Phosphorylation of Serin 392 stabilizes the tetramer formation of tumor suppressor protein p53. *Biochemistry.* 1997; 36: 10117-10124
113. Saleh-Gohari N., Bryant H. E., Schultz N., Parker K. M., Cassel T. N., Helleday T.: Spontaneous Homologous Recombination Is Induced by Collapsed Replication Forks That Are Caused by Endogenous DNA Single-Strand Breaks. *Mol. Cell. Biol.* 2005; 25: 7158-7169
114. Sancar A., Lindsey-Boltz L.A., Unsal-Kacmaz K., Linn S.: Molecular mechanisms of mammalian DNA repair and the DNA damage checkpoints. *Annu. Rev. Biochem.* 2004; 73: 39–85
115. Sarkaria J.N., Busby E.C, Tibbetts R.S., Roos P., Taya Y.,Karnitz L.M., Abraham R.T.: Inhibition of ATM and ATR kinase activities by the radiosensitizing agent, caffeine. *Cancer Res.* 1999; 59: 4375–4382
116. Scherer S. J., Maier S. M., Seifert M., Hanselmann R. G., Zang K. D., Muller-Hermelink H. K., Angel P., Welter C., and M. Schartl: p53 and c Jun functionally synergize in the regulation of the DNA repair gene hMSH2 in response to UV. *J. Biol. Chem* 2003; 275: 37469–37473
117. Schiestl R. H., Aubrecht J., Yap W. Y., Kandikonda S., and Sidhom S.: Polychlorinated biphenyls and 2,3,7,8-tetrachlorodibenzo-p-dioxin induce intrachromosomal recombination in vitro and in vivo. *Cancer Res* 1997; 57: 4378–4383
118. Sedelnikova O.A., Rogakou E.P., Panyutin I.G., Bonner W.M.: Quantitative detection of ¹²⁵IIdU-induced DNA double-strand breaks with γ -H2AX antibody. *Radiat. Res.* 2002;158:486–492.
119. Selivanova G., Wiman K. G.: P53: a cell cycle regulator activated by DNA damage. *Adv. Cancer Res.* 1995; 66: 143-80
120. Sengupta S. and Harris C.C.: p53: traffic cop at the crossroads of DNA repair and recombination. *Nat Rev Mol Cell Biol*, 2005; 6(1): 44-55

121. Sengupta S., Linke S.P., Pedoux R., Yang Q., Farnsworth J., Garfield S.H., Valerie K., Shay J.W., Ellis N.A., Wasylyk B. and Harris C.C.: BLM helicase-dependent transport of p53 to sites of stalled DNA replication forks modulates homologous recombination EMBO J 2003; 22: 1210–1222
122. Shieh S.Y., Ikeda M., Taya Y., Prives C.: DNA damage-induced phosphorylation of p53 alleviates inhibition by MDM2 Cell 1997; 91: 325-334
123. Shiloh, Y.: ATM and related protein kinases: safeguarding genome integrity. Nat. Rev. Cancer 2003; 3:155 168
124. Shono T, Tofilon PJ, Schaefer TS, Parikh D, Liu TJ, Lang FF. Apoptosis induced by adenovirus-mediated p53 gene transfer in human glioma correlates with site-specific phosphorylation. Cancer Res. 2002; 62(4):1069-76
125. Sigurdsson S., Van Komen S., Bussen W., Schild D., Albala J.S., Sung P.: Mediator function of the human Rad51B-Rad51C complex in Rad51/RPA-catalyzed DNA strands exchange. Genes Dev 2001; 15:3308-3318
126. Siliciano J.D., Canman C.E., Taya Y., Sakaguchi K., Appella E. and Kastan M.B.: Ionizing radiation activates the ATM kinase throughout the cell cycle Genes Dev 1997; 11: 3471-3481
127. Sonoda E., Zhao G.Y., Kohzaki M., Dhar P.K., Kikuchi K., Redon C., Pilch D.R., Bonner W.M., Nakano A., Watanabe M., et al.: Collaborative roles of γ -H2AX and the Rad51 paralog Xrcc3 in homologous recombinational repair. DNA Repair (Amst.) 2007;6:280–292
128. Stuerzbecher H.W., Donzelmann B., Henning W., Knippschild U. and Buchhop S.: p53 is linked directly to homologous recombination processes via Rad51/RecA protein interaction. EMBO J.1996; 15: 1992–2002
129. Subramanian, D. and Griffith, J.D. Modulation of p53 binding to Holliday junctions and 3-cytosine bulges by phosphorylation events. Biochemistry 2005; 44: 2536-44
130. Sugiyama T., Kantake N., Wu Y., Kowalczykowski S.C.: Rad52- mediated DNA annealing after Rad51-mediated DNA strand exchange promotes second ssDNA capture. EMBO J 2006; 25:5539-5548
131. Sugiyama T., Kowalczykowski S.C.: Rad52 protein associates with replication protein A (RPA)-single-stranded DNA to accelerate Rad51-mediated displacement of RPA and presynaptic complex formation, J. Biol. Chem. 2002; 277: 31663–31672
132. Sugiyama T., Zaitseva E.M., Kowalczykowski S.C.: A singlestranded DNA-binding protein is needed for efficient presynaptic complex formation by the *Saccharomyces cerevisiae* Rad51 protein. J Biol Chem 1997; 272:7940-7945
133. Tibbetts R. S., Cortez D., Brumbaugh K. M., Scully R., Livingston D., Elledge S. J., and Abraham R. T.: Functional interactions between BRCA1 and the checkpoint kinase ATR during genotoxic stress. Genes Dev. 2000 ;14:2989–3002

134. Tibbetts, R. S., Brumbaugh, K.M., Williams, J.M., Sarkaria, J.N., Cliby, W.A., Shieh, S.Y., Taya, Y., Prives, C., and Abraham, R.T.: A role for ATR in the DNA damage-induced phosphorylation of p53. *Genes Dev* 1999; 13: 152–157
135. Tripathi V., Nagarjuna T., and Sengupta S.: BLM helicase-dependent and -independent roles of 53BP1 during replication stress-mediated homologous recombination. *J Cell Biol*, 2007; 178(1): 9-14
136. Unsal-Kacmaz K., Makhov A.M., Griffith J.D., Sancar A.: Preferential binding of ATR protein to UV-damaged DNA. *Proc. Natl Acad. Sci. USA*. 2002;99:6673–6678
137. Venot C., Maratrat M., Sierra V., Conseiller E., Debussche L.: Definition of a p53 transactivation function-deficient mutant and characterization of two independent p53 transactivation subdomains oncogene 1999; 14: 2405-2410
138. Walters R.A., Gurley L.R., Tobey R.A.: Effects of caffeine on radiation-induced phenomena associated with cell-cycle traverse of mammalian cells. *Biophys J* 1974;14:99–118
139. Wang H., Boecker W., Wang H.Y., Wang X., Guan J., Thompson L.H., Nickoloff J.A. and Iliakis G.: Caffeine inhibits homology-directed repair of I-SceI-induced DNA double-strand breaks *Oncogene* 2004; 23, 824–834
140. Wang Q., Fan S., Eastman A., Worland P. J., Sausville E. A., and O'Connor P. M.: UCN-01: a potent abrogator of G2 checkpoint function in cancer cells with disrupted p53. *J. Natl. Cancer Inst.* 1996; 88: 956–965
141. Wang X.W., Tseng A., Ellis N.A., Spillare E.A., Linke S.P., Robles A.I., Seker H., Yang Q., Hu P., Beresten S., Bemmels N.A., Garfield S., Harris C.C.: Functional interaction of p53 and BLM DNA helicase in apoptosis. *J Biol. Chem.* 2001; 276 (35):32948-55
142. Wang H., Wang M., Wang H., Bocker W., Iliakis G.: Complex H2AX phosphorylation patterns by multiple kinases including ATM and DNA-PK in human cells exposed to ionizing radiation and treated with kinase inhibitors. *J Cell Physiol* 2005, 202:492-502
143. Wang, Q. E., Zhu Q., Wani M.A., Wani G., Chen J., Wani A.A.: Tumor suppressor p53 dependent recruitment of nucleotide excision repair factors XPC and TFIIH to DNA damage. *DNA Repair* 2003; 2: 483–499
144. Weinert T., Kaochar S., Jones H., Paek A., Clark A.J.: The replication fork's five degrees of freedom, their failure and genome rearrangements. *Cell Biol.* 2009; 6:778-84
145. Willers H., Dahm-Daphi J., and Powell S.N.: Repair of radiation damage to DNA. *Br J Cancer*, 2004; 90: 1297-1301
146. Willers H., McCarthy E.E., Alberti W., Dahm-Daphi J. and Powell S.N.: Loss of wild-type p53 function is responsible for upregulated homologous recombination in immortal rodent fibroblasts. *Int. Journal of Radiation Biology* 2000; 76: 1055-62

147. Willers H., McCarthy E.E., Wu B., Wunsch H., Tang W., Taghian D.G., Xia F. and Powell S.N.: Dissociation of p53-mediated suppression of homologous recombination from G1/S cell cycle checkpoint control. *Oncogene* 2000; 19: 632–639
148. Wu X., Shell S.M., Liu Y., Zou Y.: ATR-dependent checkpoint modulates XPA nuclear import in response to UV irradiation. *Oncogene* 2007;26:757–64
149. Wu X.W. Bayle H.J., Olson D., Levine A. J.: The p53-mdm-2 autoregulatory feedback loop. *Genes Dev* 1993. 7: 1126-1132
150. Yang Q., Zhang R., Wang X.W., Spillare E.A., Linke S.P., Subramanian D., Griffith J.D., Li J.L., Hickson I.D., Shen J.C., Loeb L.A., Mazur S.J., Appella E., Brosh Jr R.M., Karamakar P., Bohr V.A. and Harris C.C. : The processing of the Holliday junctions by BLM and WRN helicases is regulated by p53. *J. Biol. Chem.* 2002; 277: 31980–31987
151. Yao S-L., Akhtar A. J., McKenna K. A., Bedi G. C., Sidransky D., Mabry M., Ravi R., Collector M. I., Jones,R. J., Sharkis S. J., Fuchs E. J., and Bedi A.: Selective radiosensitization of p53-deficient cells by caffeine-mediated activation of p34cdc2 kinase. *Nat. Med.* 1996; 2: 1140–1143
152. Yonetani Y., Hochegger H., Sonoda E., Shinya S., Yoshikawa H., Takeda S., Yamazoe M.: Differential and collaborative actions of Rad51 paralog proteins in cellular response to DNA damage. *Nucleic Acids Res* 2005; 33:4544-4552
153. Yoon D., Wang Y., Stapleford K., Wiesmueller L. and Chen J.: p53 inhibits strand exchange and replication fork regression promoted by human Rad51. *J. Mol. Biol.* 2004; 336: 639–654
154. Yun S., Lie A.C.C. and Porter A.C.: Discriminatory suppression of homologous recombination by p53. *Nucleic Acids Res* 2004; 32: 6479-89
155. Zampetti-Bosseler F., Scott D.: The effect of caffeine on X-ray-induced mitotic delay in normal human and ataxia-telangiectasia fibroblasts. *Mutat. Res.* 1985; 143:251–56
156. Zhang H., Somasundram K., Peng Y., Tian H., Bi D., Weber B.L. and El-Deiry W.S.: BRCA1 physically associates with p53 and stimulates its transcriptional activity. *Oncogene* 1998; 16: 1713–1721
157. Zhao H. and Piwnicka-Worms H.: ATR-mediated checkpoint pathways regulate phosphorylation and activation of human Chk1. *Mol. Cell. Biol.* 2001; 21: 4129–4139
158. Zhou B.B., Elledge S.J.: The DNA damage response: putting checkpoints in perspective. *Nature* 2000; 408: 433–439
159. Zhou Z. H., Akgun E., and Jasin M.: Repeat expansion by homologous recombination in the mouse germ line at palindromic sequences. *Proc. Natl. Acad. Sci. USA* 2001; 98: 8326–8333

160. Zhou, J., Ahn, J., Wilson, S. H. and Prives, C. A.: role for p53 in base excision repair. *EMBO J.* 2001; 20: 914–923
161. Zink D., Mayr C., Janz C., and Wiesmüller L.: Association of p53 and MSH2 with recombinative repair complexes during S-phase. *Oncogene* 2002; 21: 4788–4800
162. Zou L., Elledge S.J.: Sensing DNA damage through ATRIP recognition of RPA- ssDNA complexes. *Science*. 2003; 300:1542–1548
163. Zou L., Liu D., Elledge S.J.: Replication protein A-mediated recruitment and activation of Rad17 complexes, *Proc. Natl. Acad. Sci. USA* 2003; 100: 13827–13832

8. Acknowledgements

I want to thank both of my supervisors, Prof. Dr. med. Jochen Dahm-Daphi at the Radiation Oncology Departments of the UKE, who accepted me as a doctorate thesis student in the first place and provided tireless help and support during the writing period, and Dr. med. Henning Willers at the Department of Radiation Oncology, Massachusetts General Hospital, for hosting and supervising the entire experimental work and for advice and teaching at any time of day. I am deeply grateful to both of them for giving me the great opportunity to conduct my experimental work abroad, and for their outstanding supervision and patience.

Further, I need to thank Lena Nitsch, Liliana Gheorghiu, Loreen Fournier, Li Li and all members of the study groups for their invaluable help and instructions during the experiments.

I am personally grateful to my parents, Christine and Otto Lachmayer, for all their help and support during my entire medschool and especially this project, to my sisters Susanne and Judith for being my role models, and to David, for everything.

9. Curriculum Vitae

Personal Information: Sarah Juliane Lachmayer,
Date and place of birth: May 6th 1983, Moenchengladbach

Medical Education: 2003 – 2005: Semmelweis Medical School,
Budapest, Hungary
2005 – 2006: University of Vienna, Medical School,
Vienna, Austria
2006 - 2010: University of Hamburg, Medical School,
Hamburg, Germany
25.11.2010: successful completion of 2. Ä.P.

Clinical Electives:
(Famulatur) 2006: Plastic and Reconstructive Surgery
St Rudolph Hospital, Vienna, Austria
2006: Internal Medicine, Diabetology and
Endocrinology, St Mary's, Hospital,
Tuebingen, Germany
2007: Gyn and Obstetrics, Nepean Hospital,
Sydney, Australia
2007: General Practice, Stuttgart, Germany

Clinical Training:
(Praktisches Jahr) 2009: Surgery, Klinikum Nordfriesland,
Husum, Germany
2009: Surgery, Nepean Clinical School,
Sydney, Australia
2010: Internal medicine, University Hospital
UKE, Hamburg, Germany
2010: Radiology, Klinikum Eilbek,
Hamburg, Germany

10. Statement of Originality

I herewith declare that I have performed the work for this thesis independently and without improper help. This work does not contain any material written or published by another person except where acknowledged. References in word or content are stated with edition, year, volume and page. I have listed all persons who directly participated in the process of this thesis. This body of work has not previously been submitted for a degree at this or any other university.

Date

Sarah Juliane Lachmayer